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C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR

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DEGENERATION AND VARIATION OF GONOCOCCI

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I. THE CULTURAL BEHAVIOUR OF DEGENERATING GONOCOCCI

In a previous paper (Casper, 1937a) we reported our investigations concerning the serological typing of 109 strains from acute cases, and some strains from chronic cases, of gonorrhea. Using two completely type-specific sera, we were able to classify a large number of the strains by means of comparative agglutination.

Several difficulties arose in the classification of the remaining strains. Some strains which we could not classify by comparative agglutination were also unclassifiable by the agglutinin-absorption test. At times, the agglutinin-absorption test gave inconsistent results even with our test strains used for immunization.

We also called attention to the fact that the cultivation of suitable test strains of gonococci is exceedingly difficult. In view of the lack of suitable culture media and of our incomplete knowledge of the optimum conditions for the cultivation of this organism, the danger of degeneration must always be considered. Experience has shown that degenerative changes in the cocci, which are not necessarily accompanied by a change in morphology, often lead to changes in their antigenic properties. Thus, the test strains with which diagnostic sera are prepared and which serve as controls in the specific absorption of strains under examination, offer numerous sources of error.

It is well known that the type-specific carbohydrate of the

pneumococcus, which is always present in organisms freshly isolated from human cases and virulent for mice, may be lost rapidly upon cultivation on artificial media. It is also known that the loss of type-specific carbohydrate is accompanied not only by a loss of virulence for mice, but also by a loss of specificity in all immune reactions. Conversely, pneumococci which by mouse-passage have become very virulent for these animals, give strongly type-specific immune reactions. Thus, we have at our disposal a means of maintaining the type-specificity of these organisms. Griffith (1928) points out that virulent pneumococci are entirely insensitive to the antibodies of the avirulent cells (R-pneumococci) and, consequently, are unable to exhaust the agglutinins from an R-serum. He also showed that the R-variant was unable to remove the type-specific agglutinins of the mother-strain. The same author (1920) was able to split off a serological variant of a type-specific meningococcus. This variant, without showing any cultural signs of degeneration, could not remove the agglutinins from a type-specific serum. He directed attention to the fact that such cultures could give rise to diagnostic errors.

Schiemann (1929) reported on the occurrence of "pseudotypes" in pneumococci and meningococci. In agar cultures of old laboratory strains of meningococci, he found a characteristic variation in colony morphology. The colonies were very small and represented a new-serological type. These strains were not agglutinated by type I or type II serum, but only by the serum of a degenerated type I strain. This "pseudotype" he never found in strains freshly isolated by lumbar puncture. Sometimes, however, it was found in strains obtained from pharyngeal cultures of meningococcus carriers. The sera of such "pseudotypes" were, naturally, unfit for the diagnosis of cases which actually occurred in practice.

Atkin (1925) attempted a classification of the gonococcus on the basis of colony morphology. He found, in fresh cultures of acute cases of gonorrhea, large papilla-bearing colonies which he designated type I. After prolonged incubation of these, a small papilla-free variant was split off. This variant he called type II.

He considered it especially noteworthy that he was able to isolate strains identical with his type II variant from cases of chronic gonorrhea. These strains were found most frequently in the cervix. According to Atkin's observations, the small papilla-free colonies are overgrown soon after isolation by the large papilla-bearing colonies but again gain predominance in aging cultures. He claimed that the papilla-free organisms were an individual pathogenic type, and assumed that classification based on colony morphology was at least as valuable as classification by serological methods. He prepared a "type I" serum with a strain freshly isolated from a male patient and his "type II" serum with an old laboratory strain showing only papilla-free colonies and originally isolated from a cervix. With these sera he was able to distinguish: (1) early forms which gave no serological reactions, but which, on further development, reacted with his "type I" serum and after the splitting off of "type II" reacted with his "type II" serum, (2) "type I" strains, (3) "type II" strains, (4) strains reacting with both "type I" and "type II" sera. A few strains were not classifiable according to his serological methods.

These investigations appear particularly interesting if one considers our observations that, in chronic gonorrhea, we found numerous strains which reacted as strongly with our type I serum as with our type II serum.

Experimental

Atkin has stated that the degenerated forms can be observed only on a semi-solid pea-broth or horse-serum agar at a pH of 7.8. While optimum conditions for growth are provided on horse serum or ascitic agar at a pH of 7.5, the gonococci tend to autolyze because of rapid growth and, as a consequence, die before the formation of papillae has begun. A pH of 7.8, on the other hand, delays growth, impedes autolysis and thus creates the conditions conducive to degeneration.

In our experiments we closely observed several strains of gonococci which had been cultivated for a long time. After testing a large group of media, we found that the best results

were obtained with our blood-water agar (Casper, 1929). On this medium, which has a pH of 7.5 to 7.6, large single papilla-bearing colonies were formed, so that it appears as if the semi-solid state plays a more important rôle than the pH. After three to five days on this medium, transplants could still be made from every portion of the colony. According to Atkin, degeneration proceeds in the following manner: At the time of cultivation on alkaline medium, a group of papillae are formed on the colony. Transplantation after five days reveals that only the papillae-bearing portions of the colony are living. Lenz and Schaefer (1936) assume that the papillae are centers of regeneration of the colony while all the other parts of the colony are dead.

We did not find dissociation of papilla-free from papilla-bearing colonies so simple. In our transplants, firstly, the papilla-free portion was not dead and, secondly, papilla-bearing colonies like those with which we started were obtained from both the papilla-free portions and from the individual papillae. In the first cultures, some completely smooth colonies were found after transplantation. These, however, proved to be non-viable, and we were not able to obtain pure papilla-free cultures in this way.

In order to obtain papilla-free colonies, we turned to culturing the papilla-free central part of the colony which, according to Atkin, is supposed to consist of confluent papillae. By this means, we obtained some colonies which produced only a few papillae after numerous transplants. As a rule, transfers were made after 4 to 5 days growth in the incubator at 37°C. By transferring only the smooth part of the colony, we eventually obtained pure papilla-free cultures. The smooth part of the colony was triturated in saline and one drop of a 1:100,000 dilution placed on a blood-water agar plate and spread with a platinum spatula, so that as many and as large colonies as possible might be obtained.

The original plate of the test strain, transplanted simultaneously, exhibited the same characteristics as on the first day. In the case of our type I gonococcus, the first transplants showed some colonies with both mesial and marginal papillae (fig. 1).

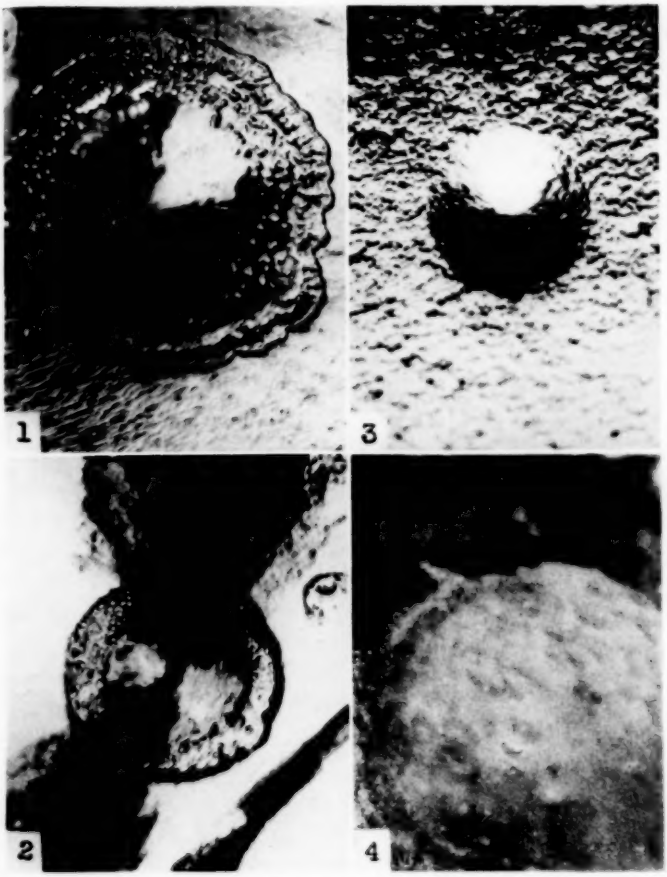

FIG. 1. SPECIFIC COLONY WITH PAPILLAE, AND FLAT, CONVOLUTED BORDERS

Papillae both marginal and central

FIG. 2. PAPILLA-BEARING COLONY AFTER CULTIVATION AND IN THE PROCESS OF TRANSFORMATION TO THE SMOOTH VARIANT

Papillae only marginal, center almost completely smooth, border no longer convoluted but almost round.

FIG. 3. TYPICAL SMOOTH FORM

Colony has no border, conic, completely smooth, no papillae. The apparent irregularities are merely the reflexion of the rough surface of the agar.

FIG. 4. VARIANT OF THE SMOOTH FORM

Colony concave (center has sunk). Border quite round. No papillae.

These papillae became very small and marginal only, then finally disappeared (fig. 2). In the subcultures, which showed only papilla-free colonies, there occurred a split into colonies of two different sizes. One, a small hemispherical (fig. 3) and the other, a more concave colony which was larger than the first (fig. 4).

Papilla-free colonies were picked out of mixed cultures for the initial injections in the production of antisera. Later the pure papilla-free cultures could be used. The procedure for preparation of the antisera has already been described (Casper, 1937a).

The transformation of our type II gonococcus culture followed a similar course. In this case, as in type I, most of the colonies also had numerous papillae and there were a few papilla-free colonies to begin with. These, however, proved to be non-viable and transplants were made from the papilla-free portion of a papilla-bearing colony. Type II also underwent several reversions to the papilla-bearing form. Strangely, there appeared large, papilla-free, staphylococcus-like whitish colonies, which, however, proved to consist of gram-negative bean-shaped diplococci. As in case of type I, we first had to use the papilla-free colonies of suitable cultures for the immunization of rabbits. Later, we obtained serviceable papilla-free cultures.

The papilla-free colonies appeared sooner than those of type I and two modifications arose, large and small smooth colonies. By subculturing, we obtained both of them in pure form.

We found no differences in the gram-staining properties of the gonococci in the original culture, in the concave papilla-free colonies, or in the large and small papilla-free colonies. We never observed those gram-positive gonococci reported by some authors (Raven 1934, etc.).

In similar fashion, a number of other gonococcus cultures which had been cultivated over a period of several months to over a year, produced both types of colonies, with and without papillae, on our blood-water agar. In every case we were able to obtain both forms in pure culture. This, however, required about three months after the original transplants had been made. We examined four each of our type I and type II and some of

our overlapping strains. The papilla-free and papilla-bearing forms of the overlapping strains were not tested serologically.

Cross-serological reactions in reciprocal sera were now undertaken with the type I strain (98) and the type II strain (116). The results of the tests of these strains, with and without papillae, and the sera obtained from them are given in table 1.

From this table it is seen that we were unable to establish those sharp serological differences between the serological types and their variants which Atkin described. We must consider, however, that Atkin produced the "type II" serum used in his diagnostic experiments with laboratory strains which had been cultivated for two years, and that he was not completely successful in verifying, by serological examination, the derivation of his "type II" from a "type I" strain. Similar to our findings, he could only describe an increase in the related reactions, which in our experiments, were most pronounced with the smooth variant.

To us, however, it seems most important that this table clearly shows that type II, as far as regards its ability to form papillae as well as its serological specificity, is to be considered as an individual type and not a derivative of type I transformed by degeneration.

Since immunization of our rabbits was begun at a time when our strains had not yet completely lost their ability to form papillae, the experiment had to be enlarged so that more exact knowledge might be gained as to how great the serological differences due to degeneration may become. Nevertheless, our results show that degenerated strains are more sensitive to overlapping antibodies.

According to the prevailing conception, we must assume that, as in the case of pneumococci, the type-specific carbohydrate masks the remaining antigenic valencies of the gonococcus.

In this experiment it would have been possible to classify our papilla-bearing type I strain on the basis of its reactions with the antisera of the degenerated variants of both types. Classification of the type II strain, however, could not have been reached on this same basis since its reactions with the sera of the degen-

TABLE 1

Cross agglutination tests of the typical (papillae-bearing) strains of types I and II and their smooth variants with the corresponding antisera

STRAINS TESTED	NORMAL RABBIT SERUM			ANTISERUM OF THE PAPILLAE BEARING STRAIN 98						ANTISERUM OF THE SMOOTH VARIANT OF STRAIN 98						ANTISERUM OF THE PAPILLAE BEARING STRAIN 116						ANTISERUM OF THE SMOOTH VARIANT OF STRAIN 116					
	25	50	100	25	50	100	200	400	800	25	50	100	200	400	800	25	50	100	200	400	800	25	50	100	200	400	800
Strain 98 type I with papillae	0	0	0	++	++	++	++	++	+	++	++	+	±	±	−	±	−	−	−	−	−	±	−	−	−	−	−
Smooth variant of strain 98	0	0	0	+	++	+	+	±	−	++	++	++	++	+	+	+	+	+	±	±	−	+	±	±	−	−	−
Strain 116 type II with papillae	0	0	0	±	−	−	−	−	−	++	+	±	−	−	−	++	++	++	++	+	+	++	++	±	−	−	−
Smooth variant of strain 116	±	±	±	+	+	±	±	−	−	+	±	±	±	−	−	++	++	++	++	++	+	+	++	++	±	±	−

erated types were appreciably different. Apparently the smooth variants differed in the degree of their degeneration. This is seen from the fact that the smooth variant of type II gave a ± reaction in 1:100 normal serum while the smooth variant of type I gave a negative reaction.

We have not investigated the question as to whether there is a specific early form without papillae, which, as Atkin assumes, is responsible for the relatively frequent lack of reactivity between freshly isolated type I gonococcus strains and their homologous sera and which, after a short period of cultivation gives a type-specific reaction. On the basis of our serological experiments, however, there is no indication for the assumption of such a form.

The use of unclassified papilla-bearing strains for the preparation of antiserum seems to assure no absolute protection against the appearance of co-agglutinins. Despite this, it should be valuable to carry out a control of the cultural behaviour, on suitable media, of the strains which are to be used for immunization and for testing the serum. Of course, the demonstration of type-specific precipitating antibodies in diagnostic sera with the aid of the specific carbohydrates (Casper, 1937b) offers far greater certainty.

Summary

Strains from fresh cases of acute gonorrhea in the male and classified as two distinct serological types were transformable from the papilla-bearing to the papilla-free form.

The formation of papilla-free colonies is a sign of degeneration probably due to growth on artificial media. Diagnostic sera prepared with such degenerated cultures are very likely to give rise to errors in classification.

Classification based only on colony morphology does not serve to exclude the possibility that we may be dealing, not with different types, but with degenerated forms of the same type which have only lost their type-specific carbohydrate.

II. THE SEROLOGICAL BEHAVIOUR OF DEGENERATED GONOCOCCUS STRAINS

The difficulties arising in the classification of freshly-isolated gonococci (Casper, 1937a) caused by non-specific reactions with type-specific immune sera have been previously indicated. In order to clarify the question as to whether the test-strains with which our immune sera were prepared had degenerated, or whether the immune sera themselves had changed, we resorted to the agglutinin-absorption test.

By means of absorption we were able to classify some strains which had given overlapping reaction with our type-specific immune sera. No definite conclusions, however, could be reached by this method with strains from chronic gonorrhea since all these strains absorbed the agglutinins of both the type I and type II sera.

It is well known that pathogenic bacteria in artificial cultures degenerate easily, and, as has been shown in the case of pneumococci, lose their ability to produce the type-specific factor. In this way they become agglutinable by the antisera of a type which does not influence the undegenerated strain. For, every pneumococcus possesses, besides the type-specific carbohydrate, factors common to all types. These common factors cannot be demonstrated because, as the cell is intact, the type-specific factor masks the reaction. In fact, due to the precipitation of these overlapping parts of the antigen, autolyzed type-specific cultures react with the antisera of heterologous types. In the same way, a partially degenerated culture might show overlapping reactions. In such cases, therefore, the types could be differentiated from each other only by absorption test. But, in an absorption test, the question always arises as to whether the overlapping reaction was caused, providing that one works with type-specific test strains and test sera, by the common protein antigen or by the presence of an individual type-specific carbohydrate related to two different type-specific factors.

We observed the serological behaviour of gonococcus test strains, previously type-specific, but which, as a consequence of

long cultivation, had undergone degeneration. A few strains had been cultivated over a period of several years. Thus, strain 1 (type I) and strain 25 (type II) were observed for six and five years respectively. From time to time, these strains were tested by agglutination in homologous and heterologous sera and also by agglutinin-absorption. Agglutination tests with one typical strain after degeneration are described in table 2.

These strains retained their specificity for a long time. Later, however, simultaneous with the drop in the titer of the homologous serum from 1:1600 to 1:400, there appeared strong agglutination in heterologous serum and also spontaneous agglutination.

TABLE 2

Changing serological behaviour of a type I gonococcus after long cultivation. Reversion to type specific agglutination by cultivation in citrated blood after apparent complete degeneration

STRAIN 1 (TYPE I)	TYPE I SERUM	TYPE II SERUM	NORMAL SERUM	SALINE
At the time of isolation, November, 1927	1:1600+	0	0	0
After cultivation, October 1930	1:400+	1:400+	1:400+	±
After transplantation to citrated guinea pig blood	1:400+	0	0	0

We were able to eliminate the spontaneous agglutination in the following manner: The culture was transplanted on 14 successive days in bouillon, to which increasing quantities (10, 25, 50 per cent) of citrated guinea pig blood had been added. This strain now gave blood water agar cultures which did not agglutinate in either saline or normal serum. At this time we found practically no co-agglutination in heterologous serum.

In table 3 are seen the results of absorption tests with strains 1a and 25a, i.e., strains 1 and 25 after degeneration. These strains were tested with antisera prepared from them while they were in the degenerated state. At the same time, they were tested with the antisera of type-specific strains.

Table 3 reveals the following facts: A degenerated strain is capable of absorbing the agglutinins from a type-specific serum

(tests 1, 2, 4, 5). Thus, it remains possible to classify type-specific strains even though degenerated strains be used for absorption.

The agglutinins of the serum prepared with a degenerated strain are not absorbed by a fresh heterologous strain (test 8). This shows that a serum prepared with a degenerated strain, which has lost most of its specific carbohydrates, may still retain some of its type-specific qualities. Although these de-

TABLE 3

Absorption tests with freshly isolated and old laboratory strains and their corresponding antisera

TEST NUMBER	TYPE SPECIFIC IMMUNE SERUM	STRAIN USED FOR ABSORPTION	EXAMINATION OF THE NON-ABSORBED SERUM WITH					EXAMINATION OF THE ABSORBED SERUM WITH				
			Type I strains		Type II strains			Type I strains		Type II strains		
			1a	76	25a	57	72	1a	76	25a	57	72
1	41 type I	1a	1,600	800	50		50	50	25	50		50
2	57 type II	1a	100			1,600		25			1,600	
3	57 type II	25a			1,600					800		
4	72 type II	25a			1,600		1,600			400		1,600
5	72 type II	25a					1,600					400
6	72 type II	72					1,600					100
7	72 type II	1a					1,600					1,600
8	1a type I	72	800					800				
9	1a type I	1a	800					200				
10	1a type I	25a	800					200				
11	1a type I	25a	400					200				
12	25a type II	25a			400					50		
13	25a type II	1a			400					50		
14	25a type II	72			400					50		

generated strains can evoke a type-specific immunological response *in vivo*, they themselves are no longer capable of reacting type-specifically. This can be seen from the overlapping reactions between the degenerated strains and their reciprocal antisera (tests 10, 11, 13).

The degree of degeneration of any particular strain may vary from day to day. In a very degenerated state, it cannot absorb the agglutinins from a type-specific serum (test 3). At such time it would be unsuitable for use as a test strain. In a more

specific state, however, the agglutinins are very strongly absorbed (test 1, 2, 4, 5). This demonstrates that a degenerated strain under certain unknown conditions may regain its type-specific factor.

Discussion

The gonococcus, like the streptococcus and pneumococcus, has been classified into several serological types. Upon cultivation on artificial media, these types undergo changes which induce the loss of their type-specific characteristics. Thus, it is seen that the behaviour of the gonococcus is quite analogous to that of the pneumococcus and streptococcus.

Investigations on the classification of streptococci (Griffith, 1934) have revealed the occurrence of certain types which are capable of absorbing the specific agglutinins from heterologous sera. Cultivation of a type-specific strain may so alter its agglutinability that it can be agglutinated, not only by its homologous antiserum, but also by a heterologous antiserum. This may occur even though there are no apparent changes in morphological or cultural characteristics. Similar observations have been made on the pneumococcus. Griffith (1928) and Schiemann (1929), have demonstrated the existence of cultural variants which have lost their type-specificity. Thus, S-organisms are not agglutinated by a serum prepared with an R-strain. It is well known that those changes which induce the loss of type-specificity of pneumococci are due to a loss of specific carbohydrate and are accompanied by a loss of virulence.

The peculiar behaviour of gonococci observed in agglutination and agglutinin-absorption tests might also be attributed to certain changes which affect their type-specificity, that is to say, the characteristic or function upon which their serological identity depends.

The loss of type-specificity which is responsible for such pronounced variations in serological behaviour is a phenomenon observed in the serology of many bacterial species which differ widely in morphology, antigenic composition and pathogenic action. In the light of these considerations it is easily under-

standable that difficulties must arise in the classification of long-cultured strains if one uses diagnostic immune sera which have not been tested on many freshly isolated strains. The fact that the so-called "pseudotype" described by Schiemann reverted into a known type after seeming to be a new one for some time, shows that freshly isolated strains must be tested in the establishment of the pathogenicity of any serologically "peculiar" type.

The loss of type-specific carbohydrate and the concurrent loss of type-specific immune reactivity is not a sudden one, but as we showed for the gonococcus (Casper 1937 b), proceeds slowly and gradually. It is, therefore, probable that at certain times a degenerating strain may possess sufficient carbohydrate for the recognition of its original type, while at other times it will give quite non-specific reactions. Along the same lines, we have seen that many reversions took place during our attempts to transform papilla-bearing to papilla-free strains, and that this transformation could only be accomplished after a very considerable number of transplants. These papilla-free forms are not capable of eliciting a type-specific immune response and the antisera prepared with them cannot be used in the classification of type-specific strains. Thus, one may say, that the loss of type-specific carbohydrate increases the ability to elicite type-specific immune response decreases. However, the 5-and 6-year old laboratory strains must have retained some of their original qualities so that at times they were able to give type-specific reactions. Whether all three changes; loss of type-specific carbohydrate, transformation to papilla-free forms and overlapping reactivity occur at the same time, we have not determined. Probability speaks for it.

If we recognize the fact that the type-specific antigenic complex is apt to be broken up before reaching the antibody-forming cells so that it becomes exceedingly difficult to prepare type-specific immune serum with them, the possibility must be considered of a change of the same degree of rapidity, but of reverse order. In other words, in the transference of gonococci, even from acute gonorrhea, to artificial media, such rapid degenera-

tion may occur that they may not only give overlapping reactions but may also be agglutinated by normal serum or saline. That these are signs of degeneration can be seen from table 2. This may account for the relatively high frequency of non-type-specific, overlapping strains, isolated from acute cases of gonorrhea.

We must now consider still another possible source of degeneration. It is known that throat cultures of meningococcus-carriers (Griffith, 1920) have yielded strains serologically different from those obtained by lumbar puncture. These strains, for the most part, are not of any definite type, but correspond to an intermediate stage, i.e., their overlapping valencies are more pronounced than those of strains from acute infections. The strains which we isolated from chronic gonorrhea never were a definite type, but always showed overlapping valencies and agglutination in saline and normal serum. Thus, we may assume that the gonococcus, by adaptation to human tissue, undergoes the same degenerative process which we see after adaptation to artificial media. This degenerative process, including the decrease of type-specific carbohydrate, carries with it a decrease of virulence. We have seen that under certain conditions type-specificity may be regained and thus may explain the exacerbation of symptom-free infections.

Summary and conclusions

1. Associated with the loss of carbohydrate in old gonococcus cultures, there is a developing relationship between formerly heterologous strains.

2. Since, at the time of isolation of gonococci from acute cases of gonorrhea, one cannot estimate the degree of their degeneration, this fact may explain the relatively high frequency of overlapping strains.

3. A theory has been advanced that in chronic gonorrhea the gonococcus, by adaptation to the human tissue, may undergo the same degenerative processes that occur after prolonged cultivation on artificial media.

III. THE SLIDE AGGLUTINATION OF TEST STRAINS OF GONOCOCCI

In view of the relatively frequent occurrence of gonococcus strains which were equally strongly agglutinated by each of two type-specific sera (Casper, 1937 a) we decided to investigate the question as to whether it was also possible to split off colonies from type-specific strains which would react with the serum of the heterologous type.

It is well known that in the cultivation of the specific phases of paratyphoid bacilli, subcultures with mixed phases are found so that unless special precautions are taken (adequate serological controls and cultivation of pure phases) most of the sera are mixed phase (Andrewes, 1922). Despite the presence of the non-specific phase in freshly isolated strains these mixed-phase sera may be sufficient for differential diagnosis, since, in these sera, the non-specific phase is "masked" and, therefore, does not exert any influence on the agglutinins of the other phase.

Bearing in mind the behaviour of the paratyphoid bacilli, we examined the antisera of our type I and type II gonococci with the assumption that, in the cultivation of these organisms, a change of phase may eradicate the serological differences between the types.

Technique of Slide agglutination: The strains tested were definitely classified by the comparative agglutination method (with the exception of the degenerated strain 25a). After diluting them with saline to 1:100,000 so that large single colonies might be obtained, the cultures were streaked on blood-water agar plates. Following the procedure used with paratyphoid bacilli and Griffith's technique with scarlatinal streptococci (Griffith, 1926 and 1927) drops of each of the concentrated sera being examined were placed next to each other on a slide and a part of a single colony emulsified in each of them. The results were read immediately as to (1) rapidity and (2) intensity of floculation. Some were read with a magnifying glass, but the majority with the naked eye. The following grades were distinguished: ++, flaky (a few large flocculi); +, flaky (several, but smaller flocculi); (+), coarsely granular (numerous fine flocculi); ±, finely granular.

TABLE 4

STRAIN	TYPE	TOTAL NUMBER OF COLONIES TESTED	SPONTANEOUSLY AGGLUTINATED	IMMUNE SERA PREPARED WITH LIVING GONOCOCCI								IMMUNE SERA PREPARED WITH KILLED GONOCOCCI															
				Type I serum 83				Type II serum 84				Type I sera								Type II sera							
												75				76				57				25a			
				++	+	±	0	++	+	±	0	++	+	±	0	++	+	±	0	++	+	±	0	++	+	±	0
76	I	10	0	10	0	0	0	0	0	2	8	0	2	1	7	0	2	1	7	0	2	1	7				
76a Degenerated mixed culture		10	5	5	0	0	0					0	(5)	0	0	0	(5)	0	0	0	2	3	0				
76b Specific subculture		13	0	13	0	0	0									0	(5)	3	5	0	0	2	11				
57 Old strain	II	12	0	0	8	0	4	0	8	0	4									12	0	0	0	0	6	0	6
25a	II	5	0	5	0	0	0	0	5	0	0									0	5	0	0	0	5	0	0

The first two occurred rapidly, showing a specific phase reaction. The last two were not so striking. In a few cases the so-called specific colonies were transferred to new plates and examined again after 24 hours incubation at 37 degrees C. Table 4 is an excerpt of numerous experiments with many different strains and their corresponding antisera.

An examination of the table reveals that strain 76 reacts specifically with serum 83 (type I). Two colonies, moreover, react quite strongly with serum 57 (type II) while one colony gives a weaker reaction. Subcultures of the former colonies yielded strain 76a. Testing of this subculture demonstrated the continued presence of mixed-phasic properties, although it could still be identified as a type I. Five of the ten colonies agglutinated spontaneously. Strain 76b was derived from that colony which gave a ± reaction with serum 57. Agglutinations done with this strain proved to be type-specific.

The presence of mixed phases is easily seen with strains 57 and 25a. Despite this fact, strain 57 can be recognized as a type II. Strain 25a, on the other hand, due to its long cultivation, has reached such a state of degeneration that it is no longer possible to classify it.

Discussion

By means of the slide-agglutination test, we were able to reveal relationships between heterologous strains of gonococci which could not be seen by a titration of agglutinins. It must be concluded from this that there may be factors which are active in concentrated sera, but which cannot be detected in diluted sera. This phenomenon appears comprehensible in view of the important rôle which the colloidal state of the medium plays in the agglutination reaction.

Let us point once more to the fact that Griffith (1926, 1937) in his investigations on the classification of scarlatinal streptococci believed that he was justified in assuming the existence of different phases of the specific types. Furthermore, let us refer to earlier experiments of Griffith (1920) in which he examined a very large number of meningococcus-carriers and discovered the

striking facts that (in contrast to their results with organisms from lumbar-puncture), in the organisms growing on the mucous membrane, type II was more frequent than type I; and that stages intermediate between both types, which seldom occur in cultures from lumbar puncture, were exceedingly frequent. At that time they assumed that they were dealing with a labile antigen which was able to develop in both directions. This theory approaches the conception of the change of phase.

Overlapping reactions in comparative agglutination have been seen, for the most part, in strains which have undergone degeneration. According to the relationships revealed by slide-agglutination, we must now consider the possibility of these relationships being due either to the loss of type-specific carbohydrate and consequent preëminence of a common protein antigen or to the existence of a carbohydrate related to each of two heterologous types (C-substance of Boor and Miller (1931)).

Atkin classified gonococci by cultural behaviour into two types. Type II, isolated from chronic gonorrhea, was designated as a papilla-free variant of his type I; the latter, isolated from acute gonorrhea, was characterized by papilla-bearing colonies. In view of the findings of Griffith and Scott with meningococcus-carriers and our experiments with gonococci, we think it advisable to consider Atkin's experience with chronic gonorrhea in a different light.

By means of comparative agglutination, we were able to classify a large number of gonococcus strains freshly isolated from acute gonorrhea into two heterologous types. We also demonstrated the existence of a completely type-specific carbohydrate for each of these (Casper, 1937b). Slide-agglutination tests performed with representative strains, on the other hand, detected the presence of mixed-phasic colonies. By picking individual colonies, we could obtain both mixed and specific phases of the same strain. Moreover, we showed that continued cultivation of a type-specific strain led to degeneration and overlapping serological reactivity due to the loss of its carbohydrate. In addition, each of the papilla-bearing type-specific strains could be transformed to papilla-free variants by prolonged cul-

tivation. Similar to the condition in carriers of the meningococcus, it is probable that chronic gonorrhea is not caused by an individual pathogenic type of gonococcus, but rather that degenerative processes like those occurring after prolonged cultivation on artificial media, may take place due to the adaptation of the organism to its environment.

Inasmuch as Atkin used old laboratory strains for the preparation of his diagnostic type II antiserum, it must be emphasized that, as the above mentioned evidence for degeneration proves, errors in classification were bound to arise.

A number of the practically observed difficulties in classification by serological methods can be accounted for by 1) preparation of diagnostic sera with organisms which were not sufficiently specific and 2) rapid degeneration of the strains immediately after isolation or accidental transplantation of a degenerated colony in purification of the culture. Whether such errors in classification can be avoided by slide-agglutination or any other method cannot yet be answered from our experiments.

Further study of variability is necessary for the solution of the difficulties of serological analysis. Many phenomena are still unexplained.

SUMMARY

1. After cultivation on artificial media, strains of gonococci which were type-specific in the comparative agglutination test were shown by slide-agglutination to have mixed-phase colonies.

2. We have discussed the possible errors in serological classification which may be caused by the use of mixed-phasic cultures.

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STUDIES ON HEMOLYTIC STREPTOCOCCI

V. The Characteristics of Human and Animal Strains of Groups A and C

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REVIEW OF THE LITERATURE

Within recent years, fermentation of trehalose and sorbitol and precipitin grouping according to Lancefield's technique have been found to indicate the human or animal source of hemolytic streptococci which formerly could not be distinguished.

Holth seems to have been the first to consider fermentation of trehalose and sorbitol for the differentiation of hemolytic streptococci. He included them in his studies together with 41 other test substances, and failed to note their significance. That discovery was made by Ogura whose data showed that lactose, trehalose and sorbitol are useful test substances for the differentiation of the streptococcus of strangles from streptococci of other animal and human sources.

Edwards extended the studies of Ogura. He studied 173 animal and 75 human strains of hemolytic streptococci. All of his human strains fermented trehalose, but not sorbitol; 159 (91.9 per cent) of his animal strains fermented sorbitol but not trehalose. Edwards concluded that sorbitol-fermenting strains of animal origin rarely, if ever, infect man.

A number of subsequent investigators have studied hemolytic streptococci in respect to fermentation of trehalose and sorbitol. Brown has recently summarized their results. He comments that in his survey of 1064 strains studied by various investigators,

[1] The junior author was responsible for the serological identification of most of the strains of groups A and C.

less than one per cent failed to be assigned to their proper serological group by fermentation of sorbitol and trehalose. Nevertheless there are in the literature reports of strains which ferment trehalose but not sorbitol and yet belong to group C. Ogura found trehalose-positive, sorbitol-negative strains among his animal strains; Edwards reported finding 7 such strains (4 per cent) among the 175 animal strains which he studied; Plummer found that among 418 strains of hemolytic streptococci which she studied, 9 (about 2.2 per cent) fermented trehalose but not sorbitol, and yet fell into group C. Hare reported that he had studied 47 strains which belonged serologically to group C although they fermented trehalose and not sorbitol. Minett reported finding 7 strains which fermented trehalose and not sorbitol among his 50 strains of hemolytic streptococci of animal origin.

Fermentation of lactose and sensitivity to phage B have also been used by previous investigators in classification studies on hemolytic streptococci. Holman used lactose fermentation in his grouping of streptococci by fermentation reactions. It was used in studies on hemolytic streptococci by Ogura; Edwards; Plummer; and Hare. Lancefield found that sensitivity to phage B filtrate was a character correlated with animal origin. In preceding papers of this series, sensitivity to nascent phage B was utilized as a character for the identification of species of groups A and C.

EXPERIMENTAL

In this paper data are presented which confirm the usefulness of trehalose and sorbitol for the identification of hemolytic streptococci of groups A and C; and data are presented to show the usefulness of sensitivity to phage B to give further information concerning strains of groups A and C. Lactose fermentation is also a useful character for the identification of certain species of those groups, although on the whole it is of less importance than the other mentioned tests.

From the results of our study of trehalose and sorbitol fermentations by hemolytic streptococci it became apparent that the usefulness of these two test substances is limited to the

differentiation of strains of Lancefield's precipitin groups A and C. (and group E, which apparently is unimportant when pathogenic strains are concerned; it will be discussed further on). Other serological groups may be differentiated from groups A and C by various cultural and biochemical tests described by Lancefield; Lancefield and Hare; Hare; Hare and Maxted; Long and Bliss; Sherman.

Group B, which commonly causes mastitis in cattle, is distinguished by its appearance on blood agar plates, by a lower final pH in glucose broth (4.2–4.8) and by hydrolysis of sodium hippurate. It is also distinguishable from groups A and C by resistance to nascent phage B.[2] The strains of group B resemble human strains of group A in fermenting trehalose but not sorbitol.

Group D (the "enterococcus") is distinguished from groups A and C by a heavier, more opaque growth on blood agar; by more vigorous fermentation of test substances including trehalose and usually also sorbitol; by a lower final pH in glucose broth (4.2–4.8) and by growth on 40 per cent bile agar. The strains of group D are also distinguishable from those of groups A and C by their resistance to nascent phage B.

Group F (the minute hemolytic streptococci of Long and Bliss) is distinguished from groups A and C by slow and scanty growth on blood agar. It is also distinguished from groups A and C by lack of sensitivity to nascent phage B. Sorbitol is not fermented; trehalose may or may not be fermented.

According to Lancefield and Hare, and Hare, group G strains resemble group A strains closely in their biochemical reactions, including the ability to digest human fibrin and to produce a soluble hemolysin. The eight strains of group G in our collection, all of which were previously studied serologically by Dr. Lancefield or Dr. Hare, were found to be resistant to nascent phage B, a character which differentiates them from the strains of groups A and C.

[2] It is unfortunate that the alphabetical designations of serological groups must be used in the same sentences with the alphabetical designations of the streptococcus bacteriophages. The phage designations bear no reference to sensitivity of serological groups.

According to Hare, who first described group H, the strains of this group may be distinguished from those of groups A and C by their distinctive appearance on blood agar, and by their inability to produce soluble hemolysin. They may be differentiated from strains of groups A and C also by their lack of sensitivity to nascent phage B, if the entire group conforms with two representative strains for which the writer is indebted to Dr. Hare. In fermentation reactions these two strains resemble the strains of groups A and C.

TABLE 1

Sensitivity to nascent phage B as a test for differentiating groups A, C and E from other groups of hemolytic streptococci

GROUP	NUMBER OF STRAINS IN THE COLLECTION*	NUMBER OF STRAINS STUDIED FOR PRECIPITIN GROUPING	SENSITIVITY TO NASCENT PHAGE B
A	362	108	+
C	191	114	+
E	3	3	+
B	44	6	−
D	28	6	−
F	10	10	−
G	8	8	−
H	2	2	−
K	4	4	−
Total	648	257	

* The grouping of some of the strains recorded in this column was by biochemical methods alone.

Group K was first described by Hare, who reported that he found only eight strains. Four of his strains were available for this study. They were found to be distinguishable from groups A and C by their resistance to nascent phage B. There is nothing distinctive in their fermentation reactions.

Table 1 summarizes the data which show that groups B, D, F, G, H and K may be distinguished from groups A, C and E by lack of sensitivity to nascent phage B. The three strains of group E, all of which were originally described by Lancefield, were isolated from certified milk. That group E is of little or no importance in studies of human streptococci is indicated by

the fact that no strains of this group were found in studies of hemolytic streptococci from human sources made by Lancefield and Hare; Hare; Hare and Maxted. None other than Lancefield's three strains were found in our collection. Through the courtesy of Dr. Lancefield, her three strains of group E were available for the present study. According to sensitivity to nascent phage B they could not be differentiated from the strains of groups A and C. They were distinguishable from the strains of those groups, however, by their ability to ferment both trehalose and sorbitol, a combination of reactions not found in strains of groups A and C (see table 3).

From the above descriptions of the characters of groups B, D, F, G, H and K, it is obvious that fermentation reactions with respect to trehalose and sorbitol are useless for differentiation, and that if these groups are included with groups A and C in differential studies by means of fermentation reactions the data are confused.

Excluding the strains of the groups mentioned, there remained 556 strains of groups A and C in our collection which was made up group by group, largely of strains already studied by other investigators. The attempt was made to obtain a sufficient number of strains of every subgroup of A and C to make a comparative study. Hence there are higher percentages of strains of the more unusual types in our collection than would be likely to be found in routine isolations.

The strains of groups A and C cannot be differentiated by their manner of growth on blood agar, nor by their final pH in glucose broth. They are alike in lack of ability to hydrolize sodium hippurate, and in lack of ability to grow on bile agar.

The data on the fermentation of trehalose and sorbitol by the 556 strains of groups A and C are summarized in table 2. Of the 409 strains of groups A and C isolated from human sources, 405 (99.0 per cent) fermented trehalose but not sorbitol. Three failed to ferment either substance; one fermented sorbitol but not trehalose. Of the three human strains which failed to ferment either substance, one was from a throat following recent recovery from septic sore throat; one was from a blister which

was a primary lesion in a case of surgical scarlet fever. The other was received with no history excepting that it was of human origin. The single human strain which fermented sorbitol but not trehalose was received from Hungary with a history of having been isolated from a scarlet fever throat. Since this strain is so unusual as a human strain, but agrees with animal strains, it seems probable that sometime there may have been some error in regard to it.

According to the data in table 2, animal strains are more diverse than human strains in their fermentation of trehalose and

TABLE 2

Fermentation of trehalose and sorbitol by hemolytic streptococci of groups A and C

FERMENTATION REACTIONS	HUMAN STRAINS		ANIMAL STRAINS	
	Number	Per cent	Number	Per cent
Trehalose − Sorbitol −	3	0.7	32	21.7
Trehalose + Sorbitol −	405	99.0	33	22.5
Trehalose − Sorbitol +	1	0.2	82	55.8
Trehalose + Sorbitol +	0	0	0	0
Total number.....	409	99.9	147	100.0

sorbitol. Among the 147 strains of animal origin with the characters of Groups A and C, 32 fermented neither substance, and 33 behaved like human strains in fermenting trehalose but not sorbitol. Eighty-two strains, or 55.8 per cent of the total number, fermented sorbitol but not trehalose.

Among the thirty-two animal strains which fermented neither trehalose nor sorbitol, 26 belonged to the species *Streptococcus equi* described in the third paper of this series.

Among the thirty-three strains of animal origin which behaved like human strains in fermenting trehalose but not sorbitol, ten were Group A strains from the udders of cows associated with

human epidemics of septic sore throat or scarlet fever. The remaining 23 were group C strains.

The most significant characteristics of the human and animal strains of groups A and C,—fermentation of trehalose, sorbitol and lactose, and sensitivity to phage B filtrate,[3]—are summarized in table 3, which includes less strains than table 2 because three odd strains were omitted. Table 3 includes all groups of two or more strains. The arabic numerals by which the groups are designated in table 3 are applied merely to facilitate the present discussion.

Table 3 shows that the majority of human strains (2) are group A strains with the characters trehalose +, sorbitol −, lactose +, phage B filtrate −. The large group with those characters includes *Streptococcus pyogenes* and *Streptococcus scarlatinae*, species which have been described in previous papers of this series, and also other subgroups.

Another smaller group of human strains (1) differs from the main group only in lack of ability to ferment lactose.

The main group of animal strains (7) is characterized thus: trehalose −, sorbitol +, lactose +, phage B filtrate +. Another important animal group (8), made up chiefly of *Streptococcus equi* strains, differs from the main group in lack of ability to ferment sorbitol and lactose.

Table 3 shows that there are in the collection a considerable number of human strains of group C which ferment trehalose but not sorbitol (4). Most of them were indistinguishable from the animal strains of the same characters not only according to the tests recorded in table 3, but also according to other tests applied, as described in the first paper of this series.

Our collection probably contains a higher percentage of the trehalose-fermenting strains of group C of human origin than would be ordinarily encountered in routine isolations in a hospital laboratory. There is evidence, however, noted further on, that they may sometimes become frequent agents of human disease within restricted localities, and for limited periods.

[3] Note that nascent phage B was used to differentiate groups A and C from other serological groups and that it is phage B filtrate which was found useful to differentiate group A from group C.

The majority of trehalose-fermenting strains of group C fail to ferment lactose (4). Our interest was directed to non-lactose-

TABLE 3

The characteristics of human and animal strains of hemolytic streptococci of groups A and C

GROUP NUMBER	HOST	NUMBER OF STRAINS IN COLLECTION	NUMBER OF STRAINS STUDIED FOR PRECIPITIN GROUPING	FERMENTATION OF			SENSITIVITY TO PHAGE B FILTRATE	NUMBER OF STRAINS IN THE GROUP	
				Trehalose	Sorbitol	Lactose		A	C
1	Human	28	28	+	−	−	−	28	0
	Animal	1	1	+	−	−	−	1	0
2	Human	320	68	+	−	+	−	68	0
	Animal	10	9	+	−	+	−	9	0
3	Human	8	7	+	−	+	+	2	5
	Animal	2	2	+	−	+	+		2
4	Human	46	35	+	−	−	+	1	34
	Animal	18	16	+	−	−	+	0	16
5	Human	0		−	−	+	+		
	Animal	3	2	−	−	+	+	0	2
6	Human	1	1	−	+	−	+	0	1
	Animal	1	1	−	+	−	+	0	1
7	Human	0		−	+	+	+		
	Animal	75	35	−	+	+	+	0	35
8	Human	0		−	−	−	+		
	Animal	29	8	−	−	−	+	0	8
9	Human	0		−	+	+	−		
	Animal	6	6	−	+	+	−	0	6
10	Human	3	3	+	−	−	−	0	3
	Animal	2	2	+	−	−	−	0	2
Total numbers...		553	224					109	115

fermenting strains because there are in the literature occasional reports of such strains being isolated from cases of human disease. According to Holman's classification they would be designated

Streptococcus equi. In the third paper of this series it was stated that the non-lactose-fermenting human strains examined did not agree with *Streptococcus equi*. Many of them, however, proved to be group C strains which fermented trehalose but not sorbitol. The human strains of group C were received from various sources, as follows:

Five strains were received through the courtesy of Dr. Griffith. They belong to three of his serological types, No. 7 (2 strains); No. 20 (1 strain); and No. 21 (2 strains).

Trehalose-fermenting strains of group C isolated from cases of erysipelas by Birkhaug were received from a number of laboratories. Birkhaug's experience with these strains is the most striking example of trehalose-fermenting strains of group C as an agent of human disease. He studied thirty-four strains of hemolytic streptococci isolated from typical cases of erysipelas. By means of the cross agglutination and agglutinin-absorption reactions he concluded that thirty-one of them belonged to one serological group. Believing that this group of streptococci was the chief agent of erysipelas, Birkhaug applied to it the name *Streptococcus erysipelatis*. Strains of this group were widely distributed in many laboratories in this country and Europe. Williams used one of them as the type strain of her erysipelas Type II, which included only six strains in her collection. In our collection of 45 strains from cases of erysipelas, the Birkhaug strains are the only group C strains. It appears, therefore, that he was misled in his conclusion concerning the importance of this group of strains as the agent of erysipelas, because he made his study at a time and place in which they happened to prevail.

Another lot of trehalose-fermenting strains of group C were received from Dr. Leonard Colebrook of Queen Charlotte's Maternity Hospital, London, England. Among the 36 strains of our collection isolated from cases of puerperal infection in the infectious ward of that hospital, 14 were trehalose-fermenting strains of group C.[4] They were all isolated during 1931 and 1932, when apparently a wave of group C puerperal infections

[4] The names of the group C strains received from Dr. Colebrook are as follows: Jackson, Nash, Hewitt, Avery, East, Steele, Tavaris, Pickerin, Amelia Martin, Mary Martin, Keen, Cutbush, Sampson and Cuzwer.

occurred in London, similar to the wave of group C erysipelas infections which occurred in Baltimore in 1925. The group C strains seem to have disappeared from the London area before Dr. Dora Colebrook made her study of 121 puerperal strains from the same infectious ward, for she found no strains of Griffith's types 7, 20 or 21.

A group of four strains of group C which fermented trehalose but not lactose nor sorbitol were received through the courtesy of Dr. Fisher, who had isolated them and five other similar lactose non-fermenting strains from cases of hemorrhagic smallpox during an epidemic in Detroit, Michigan.

Other trehalose-fermenting human strains of group C were received from scattered geographical sources and various human diseases. One was from a case of pneumonia; 1 from empyema; 1 from an abscess; 1 from a case of septicemia; 1 from a case of angina. Six were from normal throats. Two strains had histories of having been isolated from throats in cases of scarlet fever. The history of one of the scarlet fever strains (Griffith's strain "Angel," type 21) stated that it was associated with another streptococcus. It seems probable that the other group C scarlet fever strain may also have been associated with another streptococcus. At any rate there is no reason to conclude on the basis of one strain that the trehalose-fermenting strains of group C may produce an erythrogenic toxin.

The animal strains of Group C (4) which fermented trehalose but not sorbitol were from the following sources: Three were from cases of bronchitis in chicks; 5 were from cases of strangles in horses; 6 more were from other equine sources; 4 were from bovine sources, 1 of them coming from a case of acute mastitis; a few were from a variety of other sources.

Since the human and animal strains of group C which ferment trehalose but not sorbitol are indistinguishable, it appears probable that human infections with this group of streptococci may be contracted from lower animals, and *vice versa*. The occurrence of waves of human infections with trehalose-fermenting strains of Group C suggests a possible connection with bovine mastitis caused by strains of this group. The fact that certain

group C strains are capable of attacking man complicates the identification of human pathogenic strains. In routine testing, strains belonging to group C should not be disregarded as non-pathogenic for man on the basis of serological grouping alone. Their fermentation reactions should also be studied.

The five groups described above include about 95 per cent of all human and animal strains of groups A and C. The remaining 5 per cent of strains are divided among 5 small variant groups.

A study of table 3 shows that fermentation of trehalose but not of sorbitol, and resistance to phage B filtrate is a character of Group A strains regardless of host origin. Strains characterized by fermentation of sorbitol but not trehalose, and sensitivity to phage B filtrate (7), and strains negative to all 3 fermentation tests and sensitive to phage B filtrate (8), are of animal origin. Apparently they are unable to attack man. The intermediary group C strains (4) with fermentation reactions characteristic of human strains and phage sensitivity characteristic of animal strains may attack either man or lower animals.

Table 3 shows at a glance that there is no correlation between lactose fermentation and serological grouping. The usefulness of lactose fermentation in the study of groups A and C is due to the supplementary evidence afforded by the lack of fermentation of lactose, a character useful particularly for the identification of strains of *Streptococcus equi* (8) and strains of the intermediary group (4) capable of attacking both man and lower animals.

On account of the method by which this collection was built up, it would be useless to attempt to express in percentages, the distribution of groups A and C in man and the lower animals on the basis of our data. Table 3 shows, however, that among hemolytic streptococci of groups A and C the great majority of human strains belong to Group A, and that the great majority of animal strains belong to group C.

In table 4, the data on 222 strains which were classified according to precipitin grouping are arranged to show the correlation of trehalose and sorbitol fermentation and of sensitivity to phage B filtrate with serological groupings.

It is important to emphasize that the group of 222 strains

chosen for serological study included most of the strains which showed irregularities with respect to phagological or fermentation reactions on the one hand, and host source on the other hand, and that the group included only a small percentage of strains of the large groups in which all the determined characters agreed with those accepted as typical for the host species. This selection of strains was made because it was found that it is in the small irregular groups that discrepancies occur between phagological and serological reactions. Thus, only about 23 per cent of the main group (2) of human strains was examined serologically, because no irregularities were found in that group, whereas about 80 per cent of the strains of the intermediary group capable of

TABLE 4

Correlation of characters with serological grouping

GROUP	PERCENTAGE OF STRAINS WITH REACTIONS AS FOLLOWS:					
	Trehalose		Sorbitol		Sensitivity to phage B filtrate	
	+	−	+	−	+	−
A	100	0	0	100	2.8	97.2
C*	53.6*	46.4	35.7*	64.3	90.4	9.6

* See the text for the explanation of the high percentages of atypical reactions.

attacking both man and lower animals (4) were studied serologically because trehalose-sorbitol fermentations indicated relationship with group A, whereas phage sensitivity indicated relationship with group C. Almost all strains of the small groups of 10 or less strains each were included in table 4 because it is in the strains of these groups that most of the irregularities occur.

In spite of the fact that the strains included in table 4 were made up largely of those showing irregularities of one kind or another, nevertheless this selection failed to reveal significant percentages of discrepancies in trehalose-sorbitol fermentation, sensitivity to phage B filtrate and serological grouping among the group A strains. The characters of group A strains were definite,

with 100 per cent trehalose +; 100 per cent sorbitol −; and over 97 per cent resistant to phage B filtrate.

On the other hand, table 4 shows irregularity in the correlation of characters in group C. It may be pointed out that the percentages of irregularities are so magnified in table 4, by the manner of selection of irregular strains for study, that the majority of C strains included in the table were trehalose-fermenting strains. Nevertheless, even in this group selected to include a high percentage of irregular strains, 90 per cent of the C strains were sensitive to phage B filtrate. The object of the summary in table 4 was to show that among the strains of group C, sensitivity to phage B filtrate is more accurate than trehalose-sorbitol fermentation as an index to serological grouping.

SUMMARY

The serological groups A and C may be differentiated by sensitivity to nascent phage B from other groups of hemolytic streptococci with the exception of group E, which is not encountered in investigations of pathogenic hemolytic streptococci.

The present differential study includes 556 strains of hemolytic streptococci of groups A and C, 409 from human, and 147 from animal sources.

Fermentation reactions in trehalose and sorbitol broths offer useful data for the identification of strains of groups A, C and E. Lactose fermentation is also a useful character for the identification of certain species of groups A and C.

The human strains of group C and those of group A are alike in their fermentation reactions in trehalose and sorbitol broths. Of the total of 409 human strains of groups A and C, 99 per cent fermented trehalose but not sorbitol.

Animal strains of groups A and C are more diverse than human strains in their reactions in trehalose and sorbitol broths. Among the 147 strains of animal origin 32, including the 26 strains of *Streptococcus equi*, fermented neither substance; 33, including 10 strains of group A, behaved like human strains in fermenting trehalose but not sorbitol; and 82 strains, or 55.8 per cent of the total number, fermented sorbitol but not trehalose.

Group A strains are characterized by ability to ferment trehalose but not sorbitol, and by resistance to phage B filtrate. The majority of group A strains ferment lactose; a smaller subgroup differs in failing to ferment lactose.

The main subgroup of C strains is characterized by ability to ferment sorbitol and lactose but not trehalose, and by sensitivity to phage B filtrate.

Another important subgroup of C strains (*Streptococcus equi*) differs from the main group in failure to ferment all three test substances.

The strains of another sub-group of group C resemble human strains in their ability to ferment trehalose but not sorbitol and resemble animal strains in their sensitivity to phage B filtrate. They appear to be equally capable of attacking both man and lower animals.

The five mentioned groups include 95 per cent of all human and animal strains. The remaining 5 per cent of strains of our collection were divided among 5 small groups. It is in the strains of these small unimportant groups that most of the irregularities in the correlation of serological grouping with sensitivity to phage B filtrate occur.

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NUTRIENT REQUIREMENTS OF L. DELBRÜCKII IN THE LACTIC ACID FERMENTATION OF MOLASSES

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Lactic acid has been made from such materials as corn, potatoes, molasses, sugar beets, glucose, and whey by fermentation with *Lactobacillus casei*, *Lactobacillus bulgaricus*, *Streptococcus lactis*, *Lactobacillus delbrückii*, or other organisms (See references at end of paper). The manufacturer's choice of raw material depends upon his location, equipment, the price of the carbohydrate, and the quality of the product desired. Choice of culture depends upon the raw material and the conditions of fermentation selected.

Of the widely available, directly fermentable raw materials, molasses is cheap, and *L. delbrückii* is perhaps the most useful culture for the transformation of its sugar to lactic acid. We have chosen this combination and determined the conditions producing the largest yield in the shortest time.

EXPERIMENTAL PROCEDURE

The cultures, obtained from distiller's malt, were maintained on a 10 per cent malt mash, with transfers made every three or four days. Titratable acidity of this seed mash should be the equivalent of at least 15 cc. of 0.1 N acid per 10 cc. Crude cultures appeared to give as good results as pure cultures.

The inoculant for the final mashes was grown for 24 hours in molasses mash containing 5 per cent sugar, with half the usual $CaCO_3$ and a slight excess of nutrients over those of the final mashes. The latter were seeded with 3 per cent of the inoculant.

Eight liters of final mash was fermented in a 9.5 liter Pyrex

bottle equipped with a mechanical agitator, and also with a tube for moderate aeration of the fermenting mash. The theoretical amount of calcium carbonate for the complete neutralization of a 95 per cent yield of lactic acid was added at the time the mash was made up. All mashes were steamed for 90 minutes.

It was found that an incubation temperature of 50–52°C. was as high as could be used for good yields. There was no apparent trouble with infection in this range.

ANALYTICAL METHODS

Acids were determined by acidifying 300 cc. of the fermented mash with 10 cc. of concentrated sulfuric acid. After the sample had cooled and the calcium sulfate had settled, a 5-cc. clear, top sample was taken and continuously extracted with ethyl ether for 5 to 7 hours. This extraction removed both the lactic and volatile acids, which were titrated with 0.1 N NaOH and phenolphthalein as indicator. A 100-cc. sample of the clear, top liquid was taken for the volatile acid determination. It was diluted to 200 cc. in a Kjeldahl distilling flask and distilled down to 40 cc. This dilution and distillation were repeated five times. The distillate was then made up to 1000 cc., and a 100 cc. aliquot was titrated with 0.1 N NaOH. One-half of this titration was subtracted from the ether extraction titration, and the lactic acid content of the 5 cc. sample was computed from the remainder.

The accuracy of this method was tested by neutralizing 27 grams of lactic acid with 15 grams of $CaCO_3$, diluting to 300 cc., adding 10 cc. of concentrated sulfuric acid and proceeding with the analysis as with the samples. The extracts from three 5-cc. clear, top samples gave an average titration of 48.4 cc. of 0.1 N NaOH, which indicates complete recovery.

Too great an excess of sulfuric acid and a longer extraction will result in some of the H_2SO_4 being carried over with the lactic acid. Sulfuric acid should be tested for in the ether extract with barium chloride.

Fermentations were analyzed for ethyl alcohol by distilling 150 cc. from 250 cc. of fermented mash and determining the specific gravity of this distillate. However, in most cases no ethyl alcohol was found.

Residual sugars were determined by inverting a 5-cc. sample with HCl, and analyzing for reducing sugar by the macro Shaffer-Hartmann method (1921).

TABLE 1

Fermentation results

NUMBER	MASH SUCROSE CONTENT	NITROGENOUS NUTRIENT†	TIME OF FERMENTATION	RESIDUAL SUGAR AS SUCROSE	VOLATILE ACID AS ACETIC	LACTIC ACID YIELD ON SUCROSE
	grams per 100 cc.		*days*	*grams per 100 cc.*	*grams per 100 cc.*	*per cent*
1	9.20	14 per cent malt sprouts	3½	0.59	0.09	95.6
2	9.16	8 per cent malt sprouts 4 per cent steep water‡	4½	0.40	0.07	96.0
3	9.58	8 per cent steep water 10 volumes per cent thin grain residue§	6	0.30	0.07	95.0
4	9.15	14 per cent steep water	3½	0.45	0.01	94.5
5	9.28	8 per cent malt sprouts 10 volumes per cent thin grain residue	4½	0.40	0.08	95.4
6	11.22	8 per cent malt sprouts 10 volumes per cent thin grain residue	5½	0.62	0.09	94.0
7*	9.30	8 per cent malt sprouts 10 volumes per cent thin grain residue	3½	0.47	0.10	96.2
8	9.30	20 volumes per cent thin grain residue	6½	0.59	0.12	88.1
9	9.55	Nil	10½	1.98	0.23	71.3

* Blackstrap molasses; all others cane syrup.

† Expressed as per cent on weight of sucrose or as volume per cent in mash.

‡ The steep water used contained 50 per cent solids, about half of which was protein matter.

§ Thin grain residues contained 2.3 grams per 100 cc. solids.

FERMENTATION DATA

Various substances were tested as accessory nutrients for the fermentation of cane syrup: malt sprouts, steep water, thin grain residue, corn gluten meal, ammonium phosphate, ammonium sulfate, superphosphate, and beet molasses. However, the first three of these materials were found to give the best results and were used for most of the work.

Table 1 shows the data obtained with these three accessory

nutrients. The kind, amount, and combination of these accessories will influence the yield and time of fermentation. The soluble, organic nitrogen in steep water probably explains the short time of 3½ days obtained in No. 4. The blackstrap molasses used in No. 7, which also finished in 3½ days, has more soluble nitrogen than the cane syrup used in the other runs. The results of No. 8 and particularly No. 9 indicate that a lack of accessory nutrients greatly decreases the yield and materially increases the time of fermentation. These accessories may also contribute biologically useful stimulatory substances (Snell, Tatum and Peterson, 1937).

The analyses show that only traces of the fermented sugar went to other products than lactic acid. A low residual sugar and not more than a trace of volatile acid are aids in the recovery of the lactic acid.

SUMMARY

Good yields (94 to 96 per cent) and short periods of fermentation (3½ to 6 days) were obtained in the lactic acid fermentation of molasses using *Lactobacillus delbrückii* and accessory nutrients such as steep water, thin grain residue, or malt sprouts. The accessories are thought to supply soluble, organic nitrogen and biologically useful stimulatory substances.

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THE ABSORPTION OF STAPHYLOCOCCUS BACTERIOPHAGES BY ENTEROCOCCI

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The absorption of whole bacteriophages as well as of "pure line" types by the use of heat-killed susceptible and partially resistant strains of bacteria and extracts derived from such cultures has for some time attracted our attention (Rakieten, 1936, 1937). In the main, the results, paralleling those of Levine and Frisch (1934), Burnet (1934), and Sertic and Boulgakov (1935), demonstrated a marked capacity for phage absorption by susceptible organisms and their extracts. Cultures of susceptible strains of staphylococci when they were heat-killed, or of watery extracts prepared from such cultures possessed phage-inactivating action. Secondary cultures of *Salmonella enteritidis* inactivated only those types of the whole phage possessing some activity toward them. A few exceptions have been noted. Levine and Frisch, as well as Burnet, have observed the occurrence of a phage-susceptible strain whose extract had no phage-inactivating action. We have encountered a strain of *Staphylococcus albus* that absorbs bacteriophage during its growth period, but whose extract is devoid of phage-absorbing quality. Absorption of staphylococcus phages by some strains of *Bacillus subtilis* also constitutes another exception (Rakieten, 1937). The present report deals with the enterococci and their ability to absorb staphylococcus bacteriophages in spite of the fact that such organisms are not lysed by these lytic agents.

The term "enterococcus," coined by Thiercelin (1899) to designate certain Gram-positive, aerobic, fecal streptococci, now includes a fairly large group of organisms which has been defined more clearly by the studies of Andrewes and Horder (1906),

Weissenbach (1918), Dible (1921), Meyer and Schonfeld (1926), Bagger (1926), Weatherall and Dible (1929), and Sherman (1937). These streptococci are all relatively thermo-resistant, withstanding a temperature of 60°C. for thirty minutes; they grow well on media containing 10 per cent to 40 per cent bile; they ferment glucose in broth with the production of a low final pH; and they are able to split aesculin in nutrient broth as well as in media containing bile. Glycerol, maltose, mannitol, lactose, salicin, sorbitol, and trehalose are fermented; raffinose and inulin are not usually attacked. Arabinose may or may not be fermented. Methylene blue in milk is reduced. Sodium hippurate may or may not be hydrolyzed. Other criteria have been added to aid classification, but the characteristics listed above are generally deemed sufficiently inclusive to permit identification.

The 16 strains of enterococci used in this study possess in general the properties listed above.[1] Strains Z-1 and Z-11 were isolated from blood cultures of two fatal cases of subacute bacterial endocarditis. These two strains are similar in every respect to *Micrococcus zymogenes* as originally described by MacCallum and Hastings (1899), although they are not hemolytic. Strains Z-2 and Z-3 were obtained from Miss Ruth Thomas of the Department of Bacteriology, Cornell Medical School. Strains Z-4, 5, 6, 7, and 8 came from the American Type Culture Collection and correspond respectively to their *Streptococcus liquefaciens* # 4532, 4533, 4534, 4535, and 4536. They were isolated from tonsils, feces, and from the normal cervix and vagina. Culture E-9 is a beta-hemolytic Gram-positive diplococcus isolated from a normal stool. E-16 was isolated from material removed at proctoscopy from a case of ulcerative colitis; E-40 from prostatic secretion; E-68 and E-69 from enteric contents obtained during proctoscopy; E-94 from a stool culture from a case of mucus colitis, while S-9 was recovered from loose milk. The biological properties of these organisms are listed in table 1.

According to the criteria listed by Sherman (1937) the distribution of the above-mentioned strains among the possible species

[1] These strains are part of a collection of Dr. E. J. Tiffany and have been identified by him.

of the enterococcus group would be as follows: *Streptococcus fecalis*, E-16, E-40, E-9 and E-94; *Streptococcus liquefaciens*, Z-1, Z-2, Z-3, Z-6, Z-7, Z-11, and E-68; *Streptococcus zymogenes*, Z-4, Z-5, Z-8, and E-9; *Streptococcus lactis*, S-9, and possibly also E-69

TABLE 1

Biological reactions of enterococcus group organisms

STRAINS	SURVIVE MOIST HEAT		GROWTH ON MEDIUM CONTAINING			GROWS AT pH		FINAL pH IN GLUCOSE BROTH	REDUCES METHYLENE BLUE IN MILK	LIQUEFACTION OF GELATIN	PROTEOLYSIS OF MILK	HYDROLYSIS OF Na HIPPURATE	HAEMOLYSIS ON BLOOD AGAR	SOLUBLE HAEMOLYSIN PRODUCED	SPLITS ESCULIN IN BILE MEDIUM	FERMENTS WITH PRODUCTION OF ACID									
	60°C. 16 min.	60°C. 30 min.	40 per cent bile	4 per cent NaCl	6.5 per cent NaCl	9.2	9.6									Aesculin	l-Arabinose	Glycerol	Inulin	Lactose	Maltose	Mannitol	Raffinose	Salicin	Sorbitol	Sucrose	Trehalose
Z-1	+	+	+	+	+	+	+	4.6	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-2	+	±	+	+	+	+	+	4.6	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-3	+	±	+	+	+	+	+	4.6	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-4	+	+	+	+	+	+	+	4.6	+	+	+	−	+	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-5	+	+	+	+	+	+	+	4.8	+	+	+	−	+	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-6	+	±	+	+	+	+	+	4.6	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-7	+	+	+	+	+	+	+	4.6	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-8	+	+	+	+	+	+	+	4.6	+	+	+	−	+	−	+	+	−	+	−	+	+	+	−	+	+	+	+
Z-11	+	+	+	+	+	+	+	4.8	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	−	+
E-9	+	+	+	+	+	+	+	4.6	+	+	+	−	+	−	+	+	−	+	−	+	+	+	−	+	+	+	+
E-16	+	+	+	+	+	+	+	4.6	+	−	−	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
E-40	+	+	+	+	+	+	+	4.6	+	−	−	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
E-68	+	±	+	+	+	+	+	4.6	+	+	+	−	−	−	+	+	−	+	−	+	+	+	−	+	+	+	+
E-69	+	+	+	+	−	+	±	4.6	+	−	−	−	−	−	+	+	+	+	−	+	+	+	−	+	−	+	+
E-94	+	+	+	+	±	+	±	4.4	+	−	−	−	−	−	+	+	+	+	−	+	+	+	−	+	−	−	+
S-9	±	−	+	+	±	+	±	4.5	+	−	−	−	−	−	+	+	−	+	−	+	+	+	−	+	−	+	+

± = many viable organisms present but greatly reduced in numbers as compared with the non-heated control.

± = growth occurs, but is delayed and scanty.

and E-94. For testing the ability of these organisms to produce an area of hemolysis around a colony on blood agar, both human and rabbit bloods were used. Four strains were found to be hemolytic, the reaction being the same on both blood agar plates. These hemolytic organisms were also tested for their ability to produce

the so-called soluble hemolysin. The technique used was a modification of that described by Hare and Colebrook (1934). Our tests were carried out with a serum-free twelve-hour broth culture of the organisms and a suspension of washed human red cells. None of the four cultures produced a soluble hemolysin under these conditions.

The two races of staphylococcus bacteriophage used have been employed in this laboratory for other investigations dealing with absorption. Phage "V" is monovalent, lysing only culture *Staphylococcus albus* "v." Phage M-16 is polyvalent and lyses both susceptible cultures used as host strains to approximately the same titer and within the same period of time.

Susceptible culture Vnt is a hemolytic *Staphylococcus aureus* strain isolated from a case of osteomyelitis. It is susceptible to phage M-16. Culture "v" is a non-hemolytic *Staphylococcus albus* strain and is lysed by both phages. Unless stated otherwise Savita broth and agar (Rakieten, 1932) were used throughout all of these experiments, since they support the growth of enterococci as well as staphylococci.

EXPERIMENTAL

Absorption of staphylococcus phages by living cultures of enterococci

To tubes containing 10 cc. of broth, 0.1 cc. of an eighteen-hour broth culture of enterococcus and 0.05 cc. of phage M-16 was added. (The bacteriophage was so diluted that, providing no absorption took place, plaques could be counted when approximately 0.02 cc. of the filtrate was plated on susceptible cultures.) After an incubation period of from 18 to 22 hours at 35°C. the tubes containing mixtures of enterococci and staphylococcus bacteriophage, as well as the controls, were filtered through L_5 Chamberland candles. Of each filtrate 0.02 cc. was then plated for plaque counts. Plates were examined after incubation for 18 hours. Reduction in the number of plaques by an amount greater than 50 per cent was considered as positive evidence of phage absorption. The results obtained when several strains of enterococci were tested for their phage-absorbing capacity, following the procedure outlined, are listed in table 2 (A and B).

Experiments of a similar nature were carried out with strains S-9, E-69, E-94, E-68, Z-2, Z-3, Z-4, Z-5, Z-7 and Z-8 with results comparable to those listed above. From the results obtained (and many of these tests have been repeated several times using phage "V" as well as M-16) one may conclude that growing cultures of enterococci possess the ability to absorb staphylococcus bacteriophage. When cultures of enterococci that have been in contact with these phages are examined microscopically and

TABLE 2

CULTURE	BP. ADDED M-16	APPEARANCE OF TUBE	pH BEFORE FILTRATION	pH AFTER FILTRATION	PLAQUE COUNT CULTURE V
		A			
	cc.				
E-9	0.5	Turbid	5.2	5.2	1
E-9	None	Turbid	5.2	5.2	0
Z-11	0.5	Turbid	5.2	5.2	0
Z-11	None	Turbid	5.2	5.2	0
Z-1	0.5	Turbid	5.2	5.2	0
Z-1	None	Turbid	5.2	5.2	0
Broth	0.5	Clear	7.4	7.4	421
		B			
E-16	0.5	Turbid	5.2	5.2	0
Z-6	0.5	Turbid	5.2	5.2	3
E-40	0.5	Turbid	5.2	5.2	0
Broth	0.5	Clear	7.4	7.4	314

then plated no difference can be detected between them and control cultures.

It may be observed from tables 2, A and B, that all strains of enterococci produce in Savita broth a marked acid reaction. In order to assure ourselves that the presence of such a reaction in itself is not responsible for the marked diminution in corpuscular count the following experiments were carried out.

A. Absorption of phage by living cells in buffered broth

Savita broth (10 cc.) whose final pH was 7.5 was buffered by adding 0.5 cc. of 10 per cent Na_2HPO_4. (This solution was steri-

lized by filtration through L_s Chamberland candles.) To these buffered broth tubes 0.1 cc. of eighteen-hour broth cultures of enterococci along with 0.5 cc. of diluted phage M-16 or "V" were added. After an incubation period of 22 hours the reaction in all of the tubes was between pH 7.0 and 7.5. Filtrates of these culture-phage mixtures showed as marked a reduction in plaque count as those recorded with unbuffered media. This may be observed by the results indicated in table 3.

TABLE 3

Absorption of staphylococcocus phage M-16 by enterococci in buffered Savita broth

CULTURE	PHAGE M-16	REACTION pH	PLAQUE COUNT ST. V
	cc.		
E-40	0.5	7.1	1
Broth	0.5	Over 8	168
E-9	0.5	7.0	5
Broth	0.5	Over 8	217
Z-1	0.5	7.0	2
Broth	0.5	Over 8	187

B. Kinetics of absorption of phage by living cultures

In a previous report (Rakieten, 1937) it was demonstrated that a considerable amount of bacteriophage was absorbed by strains of *Bacillus subtilis* in a period of 5 hours at incubator temperature. In this connection it is interesting to note that cultures of *B. subtilis* which absorb staphylococcus phages scarcely lower the original pH of the Savita medium (pH 7.5) even after an incubation period of 58 hours. It was thought advisable to determine at what period during the growth of enterococci staphylococcus phages are absorbed. Strains Z-11 and E-40 were inoculated into broth along with the staphylococcus phage M-16. The tubes were then incubated and at the end of 5 hours and 9 hours, tubes were removed, shaken, and before filtration a pH determination was done, as well as a bacterial count. Following filtration, pH determinations and plaque counts were made. Table 4 represents the results obtained.

It is evident that culture Z-11 in a period of 5 hours at incubator temperature, and when the medium is still alkaline, is able to inactivate a considerable amount of staphylococcus phage M-16. Culture E-40, while it has approximately the same rate of growth as has strain Z-11, takes a little longer to bring about a significant reduction in plaque count. On the basis of the evidence obtained in the above experiments it is believed that the absorption of staphylococcus phage by enterococci is not related to a change in the reaction of the medium brought about by the growth of these cultures.

The results obtained thus far lead us to believe that enterococci have some protoplasmic component in common with staphylococci, in that it is able to combine with and absorb staphylococcus phages. The antigenic component of susceptible strains of staphylococci and cultures of *B. subtilis* which absorbs staphylococcus phages is remarkably heat stable, since autoclaved cultures possess this phage-inactivating quality. Hence, it was desirable to ascertain whether heat-killed cultures of enterococci still retained the absorbing power. Cultures of Z-11, E-40, Z-8, E-69, E-9, E-16, Z-4 and Z-7 were heated for one hour at 62–63.5°C., the organisms were resuspended in fresh broth and then staphylococcus phages were added. Following incubation the mixtures were tested for the presence of phage by plaque count. In no instance did we find that there was any significant reduction over that of controls. In this respect the component in these

TABLE 4

CULTURE	BP. ADDED M-16	INCUBATION PERIOD	REACTION pH	BACTERIAL COUNT	REACTION FOLLOWING FILTRATION	PLAQUE COUNT
	cc.	hours		0.1 cc. 10^8		
Z-11	0.5	5	7.1	1018	7.1	41
E-40	0.5	5	7.0	994	7.0	175
Broth	0.5	5	7.5		7.5	234
				0.1 cc. 10^8		
Z-11	0.5	9	7.1	124	7.0	18
E-40	0.5	9	6.8	76	6.8	1
Broth	0.5	9	7.5		7.5	204

strains responsible for phage absorption is much more heat labile than it is in staphylococci. However, a bacteriophage ("Zy") which lyses these strains of enterococci is absorbed by these same heat-killed cultures. This latter finding is in keeping with the results obtained by other workers dealing with the ability of heat-killed susceptible cultures to absorb homologous phages.

Cultures were killed by other means in an attempt to learn whether such cultures would absorb homologous phages. Commercial formalin in a quantity sufficient to bring the final concentration to 0.5 per cent was added to fifteen-hour cultures of the organisms in plain infusion broth. Following a twenty-four-hour incubation period they were centrifuged, resuspended, and washed three times with physiological saline. After a third washing the organisms were resuspended in saline; sterility tests showed no viable organisms.

Cultures Z-1, Z-11, E-9, and E-40 were subjected to the action of formalin in the manner indicated above. To 5.0 cc. quantities of the washed sediments, resuspended in saline, as well as to the third and final saline washings of each culture, 0.5 cc. of staphylococcus bacteriophage "V" and enterococcus phage "Zy" were added. After incubation for 20 hours a portion of each mixture was overlaid on susceptible staphylococcus "v" and enterococcus E-40, in order to obtain plaque counts. The results appear in table 5.

That the bacteriophage inactivating effect of formalin-killed enterococci was not due to the presence of formalin is evident, since in no instance did the final wash-fluid reduce the plaque count. Toward the homologous active phage, formalinized cultures have a considerable degree of phage inactivation power. With a staphylococcus bacteriophage that is markedly inactivated by these cultures in their actively proliferating state, these same formalinized cultures cause a significant reduction in corpuscular count.

Enterococci killed by another method also retain their staphylococcus phage-inactivating power. This method entails grinding the bacteria in an apparatus similar to that employed by Mak (1933). Our apparatus was constructed so that two tubes of

culture might be ground at the same time. Pyrex tubes 3.5 x 17.5 cm. containing approximately 150 quarter-inch stainless steel balls revolving at a speed of 300 revolutions per minute constituted the grinding chamber.

Cultures of staphylococcus Vnt and enterococcus E-40 were grown in Roux bottles. After an incubation period of 24 hours the growth in each bottle was washed off with 5 cc. of sterile distilled water. The mixtures were then placed in the sterile grinding chamber and after 48 hours of continual grinding (average temperature 20°C.), the material was removed and examined

TABLE 5

Absorption of staphylococcus and enterococcus phages by formalinized enterococcus cultures

CULTURE 5.0 CC.	THIRD WASHING FLUID 5.0 CC.	BP. ADDED "ZY"	BP. ADDED "V"	PLAQUE COUNT	
				E-40	Sta. v
		cc.	cc.		
Z-11		0.5	0.5	0	76
	Z-11	0.5	0.5	Cfl	200
Z-1		0.5	0.5	0	101
	Z-1	0.5	0.5	Cfl	200
E-9		0.5	0.5	109	81
	E-9	0.5	0.5	Cfl	200
E-40		0.5	0.5	0	91
	E-40	0.5	0.5	Cfl	200
Broth		0.5		Cfl	
Broth			0.5		217

microscopically and culturally. In the case of the staphylococcus, only Gram-negative amorphous material was observed. The enterococcus material revealed on smear relatively few organisms retaining their Gram stain. However, on culture, viable cells were found. In order to free this ground suspension of most of the viable cells it was centrifuged and the supernatant removed. These supernatant fluids, in 0.5 cc. quantities were added to 0.5 cc. of bacteriophages "Zy" and "V." These mixtures were incubated at 20°C. for 24 hours, and without filtering were tested for their ability to produce plaques. A portion of each supernatant was also heated for one hour at 63°C., to these

fluids, equal quantities of phages were added, and following incubation at 20°C. they also were plated for plaques. The results are listed in table 6.

Similar results were obtained with ground material from enterococcus culture E-40. These ground preparations were kept in the ice-box for three weeks and again tested for their phage-inactivating power. No decrease in absorbing quality was observed. Toward homologous phages, material from heated as well as from unheated ground cultures has very strong absorbing power. Ground cultures of enterococci that have been heated lose this staphylococcus phage-inactivating ability. This result is consistent with that obtained when whole cells have been

TABLE 6

SUPERNATANT GROUND CULT.	BP. M-16	PLAQUE COUNT	SUPERNATANT GROUND CULT.	BP. "ZY"	PLAQUE COUNT
	cc.			cc.	
Vnt. unheated	0.5	4	Vnt. unheated	0.5	150
Vnt. heated	0.5	0	Vnt. heated	0.5	Cfl
E-16 unheated	0.5	161	E-16 unheated	0.5	0
E-16 heated	0.5	400	E-16 heated	0.5	0
Broth	0.5	400	Broth	0.5	Cfl

subjected to heat. Ground cultures of staphylococcus Vnt, unheated, also absorb enterococcus phage "Zy."

During the course of this study we have also tested three other susceptible strains of staphylococcus for their enterococcus phage-absorbing ability. In all instances positive evidence was acquired. Also, cultures of *B. subtilis* which previously had been shown to absorb staphylococcus phage will also absorb enterococcus phage "Zy." Enterococcus cultures E-16, E-40, and Z-11 will also absorb to a high degree *B. subtilis* phage C-3. Pneumococcus types I, II, and III when they were tested for their phage absorbing activity had no demonstrable effect on our staphylococcus phages or enterococcus phage.

Extracts of strains E-16, E-40 and Z-11 were prepared according to the method originally described by Burnet (1934) in order to determine whether the component responsible for staphylococcus

phage-absorption is present in such preparations. The clear watery extracts of the above mentioned cultures were added in 0.5 cc. amounts to staphylococcus phages. After an incubation period of 20 hours at 35°C., approximately 0.02 cc. of each unfiltered mixture was overlayed on agar-plate susceptible cultures, and the plates later observed for plaque counts. Extracts prepared and tested in this fashion do absorb homologous phages, but have no demonstrable effect on staphylococcus phages. Protocol 7 illustrates one of the results obtained.

No differences in results were noted when staphylococcus phage M-16 was used. It is obvious that extracts of enterococci

TABLE 7

Watery extracts from cultures E-40, E-16, Z-11 and their effect on homologous and staphylococcus phages

EXTRACT (0.5 CC.)	BP. 0.5 CC.	PLAQUE COUNT
		E-16
E-40	"Zy"	109
E-16	"Zy"	0
Z-11	"Zy"	11
Distilled water	"Zy"	Cfl
		Sta. v
E-40	"V"	Over 400
E-16	"V"	Over 400
Z-11	"V"	Over 400
Distilled water	"V"	Over 400

prepared in this fashion lack phage-inactivating action toward staphylococcus phages. Since these extracts are prepared by allowing the cultures to autolyze at 57° to 58°C. for 48 hours it may be that this degree of heat is responsible for the loss in absorbing effect, since heating living as well as ground cultures at a temperature only slightly higher destroys the absorbing effect toward these same phages.

Attempts, using serological methods, have also been carried out in order to learn whether the component in enterococci that absorbs staphylococcus phages is similar to staphylococcus protein. These tests are still being followed, but thus far they have given no conclusive evidence that precipitin produced by in-

jecting rabbits with formalinized enterococci reacts to any considerable degree with staphylococcus precipitinogen (protein fractions). These same fractions react with homologous precipitin to a high titer. This portion of the study will be reported at some later date in greater detail.

Finally, protein and carbohydrate fractions of enterococci and staphylococci have been prepared in order to see whether these fractions will absorb homologous and heterologous phages. The methods used to isolate these protein and carbohydrate preparations were similar to those reported by Lancefield (1925) for the streptococci.

The organisms grown in Roux bottles on the surface of extract agar, containing 0.1 per cent glucose, were suspended after 48 hours' incubation in a small volume of sterile distilled water. The organisms were washed twice with sterile distilled water, once with 95 per cent alcohol, and once with ether, and dried in a vacuum desiccator. The dried mass was ground in the ball-mill for 12 hours, at the end of which time there were no intact organisms to be found in a Gram-stained smear. N/100 NaOH did not suffice to bring the material into complete solution and we were forced to resort to N/50 NaOH. To this solution 10 per cent acetic acid was added until precipitation was complete. The precipitate was then centrifuged and the supernatant decanted and saved. The precipitate was redissolved in N/50 NaOH and reprecipitated with acid. This process was repeated three or four times and the acid supernatants saved and pooled. The precipitate was finally washed several times in water and dried in the vacuum desiccator. This product was labeled "P" A.

The combined acid supernatants were evaporated to about 1/10 volume in a stream of warm air, acidified with acetic acid in the cold, any precipitate discarded, and the acid solution kept in a boiling water bath for 10 minutes to precipitate coagulable protein, and then centrifuged. To the clear supernatant was added slowly ten volumes of 95 per cent alcohol. The resulting precipitate was dissolved in 5 to 10 cc. of distilled water. This solution was reprecipitated with alcohol and redissolved several times. The final precipitate was dried in the vacuum desiccator and labeled "C" A.

Other mass cultures of these organisms, grown in the same way, were treated according to the method of Avery and Goebel (1933) in the preparation of acetyl polysaccharide from the pneumococcus. For this fractionation the surface growths from twenty-seven Roux bottles were used, amounting to 270 cc. of bacterial suspension. This represented the accumulated harvests over a period of ten days, and was stored in the ice-box in the meantime, with the addition of 1/10,000 merthiolate to prevent contamination. To this suspension was added about 80 cc. of N/1 acetic acid and the mixture was heated in the Arnold sterilizer at 100°C. for thirty minutes. Upon cooling, the bacteria settled to the bottom of the flask in a granular precipitate. After standing in the ice-box overnight, the bacteria, plus any coagulated material, were removed by centrifugation and the supernatant, supposedly containing the polysaccharide, was stored in the ice-box. The sediment was washed twice and the washings added to the supernatant. The sediment was washed with alcohol, ether, then dried, ground in the ball-mill, dissolved in N/50 NaOH, and precipitated several times with acetic acid. The final dry precipitate was labeled "P" B.

The combined washings and supernatant were evaporated to 1/10 volume and to the solution was added slowly an equal volume of 94 per cent alcohol. The heavy flocculent precipitate was removed by centrifugation and dissolved in about 70 cc. of distilled water. This solution was acidified with N/1 acetic acid and the fine precipitate discarded. The solution was again precipitated with an equal volume of alcohol, and the precipitate redissolved in water. Precipitation and solution were repeated twice, the final solution, acidified with N/5 HCl, was dialyzed through cellophane until no Cl^- ions were demonstrable in the dialysate. The solution was then precipitated with ten volumes of acidulated acetone and the precipitate recovered by filtration. This product was labeled "C" B.

All "C" fractions gave strong positive Molisch reactions, and negative Millon and xanthoproteic reactions. We realize that the preparation of polysaccharide fractions of these organisms by the method which Avery and Goebel used to obtain an acetyl

polysaccharide from the pneumococcus does not prove that our fractions "C" B are acetylated polysaccharides. At this time we merely report our findings with polysaccharide fractions of these organisms, obtained by two different methods. The "P" fractions gave positive Millon and xanthoproteic reactions, but also positive Molisch reactions, indicating that these fractions were not free from carbohydrate.

Equal quantities of carbohydrate fractions and diluted bacteriophages (0.03 cc. of each) were placed in small tubes and incubated for twenty-two hours at 35°C. A portion of each mixture was then overlayed on agar-plate susceptible cultures and later examined for plaques. None of the carbohydrate

TABLE 8

	PLAQUE COUNT
Heat-killed staph. Vnt. with Bp. M-16	0
Heat-killed staph. v with Bp. M-16	0
Acid-treated staph. Vnt. with Bp. M-16	260
Acid-treated staph. v with Bp. M-16	260
Distilled water with Bp. M-16	260

fractions "C" A, or "C" B, had any demonstrable inactivating effect, either on homologous or heterologous phages. These results are in accord with those published by Gough and Burnet (1934).

That a carbohydrate associated with the somatic antigen may play some rôle in the ability of such antigens to absorb phages may be concluded from the following experiments. Agar-slant cultures of staphylococci were washed off with sterile distilled water. The resulting suspensions were then heat-killed by autoclaving. Each suspension was divided equally, one portion being retained as a heat-killed suspension, while the other was boiled in N/20 HCl for twenty minutes. The acid-treated suspension was then washed, resuspended in saline, and neutralized. To both the heat-killed and acid-treated suspensions equal amounts of staphylococcus bacteriophage were added and the mixtures incubated for eighteen hours at 35°C. Portions (0.02 cc.) were then

tested by our usual technique for the presence of plaques. That heat-killed cultures that have been treated with acid lose their phage-inactivating effect may be observed from the results shown in table 8.

Protein fractions, "P" A, and "P" B, of enterococcus strains E-16, Z-1, Z-11, and E-9 and staphylococcus cultures "v," Vnt, and pg were tested for their phage-absorbing ability; 0.5 cc. of each fraction was added to 0.5 cc. of phage and the mixtures incubated for twenty-two hours at 35°C., when 0.02 cc. quantities

TABLE 9

	PLAQUE COUNT
"P" A of culture E-16 with Bp. "Zy"	1
"P" A of culture Z-1 with Bp. "Zy"	0
"P" A of culture E-9 with Bp. "Zy"	0
"P" A of culture Z-11 with Bp. "Zy"	0
Saline with Bp. "Zy"	Over 200
"P" B of culture E-16 with Bp. "Zy"	2
Saline with Bp. "Zy"	311
"P" A of culture v with Bp. M-16	148
"P" A of culture Vnt. with Bp. M-16	0
"P" A of culture pg with Bp. M-16	0
Saline with Bp. M-16	161
"P" A of culture E-16 with Bp. M-16	87
"P" B of culture E-16 with Bp. M-16	134
Saline with Bp. M-16	312

of each mixture were plated for plaques against susceptible cultures. The results are listed in table 9.

The protein fractions from cultures of enterococci show as strong an ability to absorb homologous phage as do heat-killed and formalin-treated whole cultures. The protein fractions of culture E-16 also possess a significant amount of staphylococcus phage-inactivating action. Our results with the protein fraction of staphylococcus v run counter to those obtained with heat-killed cultures and watery extracts from autolyzed cultures. On three occasions we have prepared protein fractions of this culture and in each instance the fraction has had no phage-inactivating action toward staphylococcus phages M-16 and "V." We are

unable, at this time, to give an adequate explanation for this exception. Protein fractions of other susceptible cultures of staphylococci behave with regard to their phage-inactivating action in a fashion similar to heat-killed cultures and extracts prepared from these strains.

DISCUSSION

The principle that bacteriophages, in general, are absorbed only by those strains of bacteria (heat-killed, or their extracts) that are visibly affected by contact with these lytic agents is well founded. Most of the facts, however, upon which this concept has been built have resulted from studies dealing with phages that attack the enteric group of bacteria. This large group of lytic agents is very slightly affected by such non-bacterial agents as pus, spinal fluid, whole blood, and serum. The staphylococcus bacteriophages are quite different. Most of them are weakened very considerably by contact (*in vitro*) with the same non-bacterial substances that ordinarily do not influence the action of phages which attack Gram-negative bacilli. They also differ in that they are absorbed by bacteria against which they have no demonstrable effect. Evidence has been obtained, however, to correlate the absorption of staphylococcus phages by organisms of the *Bacillus subtilis* group with the presence of staphylococcus-like antigen in these bacilli (Rakieten 1937).

The enterococci constitute another group of organisms which have a demonstrable inactivating effect on staphylococcus phages, in spite of the fact that contact with these lytic agents produces no observable changes in these microorganisms. On the basis of the results obtained with living cells, cultures killed by formalin and by grinding, we have concluded that enterococci have some antigenic component (surface?) which behaves like the surface antigenic component from susceptible staphylococci. That this component is distinctly heat-labile may be deduced from the fact that heated whole cultures, heated ground cultures, and watery extracts do not possess power to inactivate the staphylococcus phage. If this component is a surface one, then it must be present in very low concentration, since all of our at-

tempts to produce precipitin which will react with staphylococcus precipitinogen have consistently failed. Since all of the rabbits we have employed had a high titer of naturally occurring agglutinin for staphylococci and a moderately high titer for enterococci, any increase in this antibody using heterologous antigen would be difficult to assess. No naturally occurring precipitin was found, and the production of this antibody by whole cells as well as by variously prepared protein fractions is still being attempted. If further study demonstrates that the staphylococcus-like component in enterococci is not detectable by ordinary serological methods, the value of phages for this purpose is very obvious.

The enterococci are unlike the three fixed types of pneumococci, studied by us, for we have been unable to demonstrate that these cocci consistently inactivate staphylococcus phages to any significant degree.

The results with the protein fractions of enterococci strengthen the view that these organisms have a fairly close relationship to staphylococci. Many more of these fractions are being studied, especially since fractions prepared from the same strains on different occasions differed in their phage-absorbing capacity.

Finally it becomes more and more evident why staphylococcus phages are so difficult to obtain from stool filtrates as well as from sewage filtrates. Apparently, diverse microorganisms are able to inactivate these lytic agents, making isolation uncertain from so-called rich sources of bacteriophage.

CONCLUSIONS

1. Actively growing cultures of enterococci possess a considerable power to inactivate staphylococcus bacteriophage.

2. Formalin-killed cultures as well as ground cultures also possess the same degree of activity.

3. Heat-killed whole cultures as well as heated ground culture material lose their ability to inhibit staphylococcus phages.

4. Watery extracts of enterococci absorb homologous phage, but have no demonstrable staphylococcus phage inactivating activity.

5. Staphylococci as well as some strains of *Bacillus subtilis* absorb an enterococcus phage.

6. Pneumococci, types I, II, and III, do not affect staphylococcus phages.

7. Carbohydrate fractions of enterococci are devoid of phage inactivating activity.

8. Protein fractions of these same strains will absorb homologous phage and to some extent heterologous phage (staphylococcus).

9. The component in enterococci responsible for staphylococcus phage inactivation can not be demonstrated, consistently, by the use of a serological method (production of precipitin).

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MICROBIOLOGY OF THE UPPER AIR

III. An Improved Apparatus and Technique for Upper Air Investigations[1]

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Research in the microbiology of the upper air at the Massachusetts Institute of Technology was inaugurated in 1932. During the past five years, airplane collections of microorganisms and other air-borne materials have been made, in the course of more than two hundred separate flights in the vicinity of Boston. The first instrument used at the Massachusetts Institute of Technology was described by one of us (Proctor, 1934) together with certain of the findings made previous to that time.

Workers in this and other countries have been engaged in somewhat similar researches which have added a number of different devices capable of use for such collections, both from airplanes and balloons. Among the devices of more recent origin are those described by Lindbergh and Meier (1935), and Rogers and Meier (1936) in the United States, and Van Overeem (1936) in Holland.

The present paper is concerned with modifications in the bioaercollector and in the laboratory technique involved in its use which minimize the opportunity for contamination during collection and examination. The modifications also permit photographic records under aseptic conditions of the non-viable particulate matter which comprises the major portion of the collections, thus allowing subsequent culture of the living material collected.

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The collecting unit consists of a solid brass disc (plate 1, fig. 2) containing six holes in which collecting slides are held in small brass collars. Between these six holes are six smaller by-pass holes through which the air-stream flows, between collections. The collecting slides are circular 18 mm. no. 1 coverslips. A collar with a collecting slide in place is shown in plate 2, figure 6. The diameter of the collector holes is 20 mm., and the air-stream passes around the collecting slide through the 1 mm. annular space left when the collecting slide is in place. The brass disc is enclosed in a brass case (plate 1, figs. 1 and 3) and can be rotated by hand wheel (plate 1, fig. 1) to line up each of the collecting slides successively with the air intake. The case and disc have been carefully machined so that the flat surfaces fit tightly together. In addition, the contacting surfaces are covered with a heavy mineral oil. As a result of these precautions and the spatial arrangement of the collector holes, there appears to be no opportunity for contamination between collector holes or any chance of air flowing through more than one collector hole at a time. The air intake consists of a straight brass pipe with a double protecting collar which can be opened or closed by a sliding rod on the side of the collecting pipe. The air to be sampled enters through the top of the collecting pipe, passes down through the straight pipe, impinges directly upon the oiled collecting slide at right angles to the plane of the glass, flows around the slide through the annular space and out through the suction end of the collector. Air circulation through the device is obtained by a two-inch Venturi tube mounted on a strut of the plane and connecting with the collector by a rubber tube. The assembled apparatus is shown in plate 1, figure 4.

The equipment is prepared for use in the following manner: The disc and the inside walls of the enclosing case are cleaned and oiled with a heavy, clear, colorless mineral oil. Cover slips cleaned and previously stored in 95 per cent alcohol are flamed and placed in the holders which are then fixed firmly in the disc. Filtered mineral oil is placed upon these cover slips on the side facing the air intake, thereby constituting an adhesive surface

upon which the air impinges. The retained material is the source of our collections. The upper surface of the cover slip is nearly 1 mm. below the top of the collector hole so that oil from the case will not be drawn by capillarity on to the slide and thus contaminate the specially cleaned oil on the collecting slide. The apparatus is then assembled as a unit and sterilized in a hot air oven at 180°C. for two hours.

The collector is mounted in the cabin or cockpit of the plane by means of a circular clamp, so that the collector pipe extends outward into the slipstream of the propeller. The protective cap prevents dust from entering the collector during flight preparations and take-off. At an elevation of 1500 feet the protective cap is opened and a ten-minute sample taken by allowing air to be drawn in over the oiled cover slide.[2] The brass disc is turned to the next hole which is a by-pass opening and left for a period of one minute, after which the disc is again turned to the succeeding collector hole where another collection is made in the same manner as before. Following this same routine the remaining slides are exposed, with an interval between each exposure of a one-minute flow of air through each successive by-pass hole, thus permitting an air wash. At the end of the fifth exposure, the tightly fitting protective cap at the exterior end of the collecting pipe is manually closed from within the plane.

As stated previously, there are six collecting slides (cover slips) mounted in the disc. The sixth slide is not exposed; it serves as a control, and the device is so arranged that this slide cannot be lined up with the air intake. When examined with the other slides, this control slide serves as a reliable index of imperfect sterilization or contamination.

During the past two years our equipment has been flown and operated by army pilots in the Boston weather plane. These flights were made to an altitude of 16,500 feet as the maximum

[2] The rate of air flow through the instrument is approximately 1.1 cubic feet per minute and was determined by laboratory calibration of the pressure differential at average flight speeds. A larger instrument with a greater air flow and more numerous collecting slides is now under construction.

elevation. On this schedule five samples were taken from 1,500 feet to 16,500 feet; each sample being taken during a 3,000 foot climb.[3]

Examination of the collected material is carried out in the following manner: The inlet side of the case (plate 1, fig. 3) is removed and the exposed side of the disc is quickly placed upon a brass plate (plate 2, fig. 5) on which three sterile microscope slides are arranged. The oil on the disc causes the microscope slides to adhere and cover the six collector holes when the disc is lifted. The other half of the case is then removed, the disc is inverted and placed upon a similar brass plate (plate 2, fig. 7). The holes on the other side are covered in the same manner by protective microscope slides. As a result the collector holes on both sides of the disc are protected against contamination.

The disc, containing protected collecting slides, is placed upon the pedestal plate (plate 2, fig. 8) which is then mounted in a modified mechanical stage of a microscope. The disc may be rotated on this plate so that the protected collector slides may be examined and photographed. Representative areas are then selected and photographed on 35 mm. motion picture film, thereby yielding permanent records. Calibrations of the microscope and camera permit quantitative comparison of the collected material.

After examination and photography are completed the protective slides on the under side of the disc are removed. Each upper protective slide is moved sidewise to expose one hole at a time and the corresponding collecting slide holder is moved upward to allow removal of the collecting slides. Each collecting slide is transferred by sterile forceps to a sterilized four-ounce rubber-stoppered bottle which contains 7 m.l. of sterile distilled water. After the bottles have been shaken, melted standard nutrient agar[4] of double concentration is added and mixed. The

[3] Previous to this coöperative arrangement between the U. S. Army Air Corps and the U. S. Weather Bureau, collections were made during the course of flights to heights over 20,000 feet by a meteorological plane owned and operated by the Massachusetts Institute of Technology. The results of these flights were reported in the Journal of Bacteriology (Proctor, 1935).

[4] Other specialized types of culture media have been used for the development of particular types of microörganisms.

bottles are slanted and incubated at 20°C. and 37°C. for several days at each temperature, after which the organisms are subcultured and identified.

If any microorganisms develop from similar treatment of slide 6, the control, the collections of the entire flight are rejected. The heat sterilization of the intact collector previous to use and the oil seal between disc and case, combined with the closure mechanism of the inlet orifice, appear to prevent contamination. Only in rare instances has any growth been found from the control slide.

The possibility of dust or organisms being temporarily retained in the collector pipe and dislodged to contaminate a succeeding collection has been minimized by covering the inner surfaces of the collecting pipe and the protective cap assembly with the same heavy mineral oil used in the collector itself. Particles striking these oiled surfaces adhere in the same way as they do on the collector slides. From an aerodynamic viewpoint, the air velocity close to these surfaces is much lower than that in the major internal portion of air stream. Therefore it is considered that the possibility of dislodging these trapped particles is very slight.

As the inlet end of the collector pipe is in the slip-stream of the propeller, there is a possibility that materials originating on the motor housing or the propeller assembly could enter the collector. To minimize this type of contamination, the oiled protective cap is kept closed during the time preparatory to take-off and until the plane has reached an elevation of 1500 feet. Air flow tests indicate a relatively high velocity close to these surfaces. This results in a substantial air wash of nearly thirty minutes previous to the time the collector is opened, which would seem to be a sufficient interval to dislodge from the smooth, highly polished surfaces of the plane most materials which might later influence the collections. The fact that in numerous instances we have found no viable organisms in an entire collection would seem to preclude the possibility of gross contamination from such surfaces. Until such time as our equipment may be mounted anterior to the propellers, this possibility of contamination cannot be entirely excluded.

Since the start of this work, a large number of bacteria and molds have been collected and identified. Using the same improved apparatus, just described, forty flights were made over greater Boston from November, 1936 through June, 1937.

TABLE 1

HEIGHT	BACTERIA*	MOLDS†
feet		
1,500–4,500	Achromobacter rathonis Bacillus cereus	Aspergillus fumigatus Aspergillus niger Aspergillus flavus Macrosporium sp. Penicillium frequentans
4,500–7,500	Bacillus ruminatus Bacillus aerosporus Bacillus simplex Bacillus albolactis	Aspergillus niger Aspergillus glaucus Cladosporium sp.
7,500–10,500	Sarcina lutea Bacillus megatherium Bacillus prausnitzii Bacillus albolactis	Aspergillus niger Hormodendrum herbarum
10,500–13,500	Bacillus cereus Kurthia zopfii	Aspergillus calyptratus Hormodendrum herbarum
13,500–16,500	Micrococcus candidus Bacillus subtilis Bacillus albolactis Bacillus simplex	Penicillium glabrum Penicillium lanosum

* Bergey's Manual, edition 4, was used for identification.

† The authors are indebted to Dr. David H. Linder of the Farlow Herbarium, Harvard University, for assistance in the identification of certain of the molds.

Photographic records have been made of the particulate material collected during these and some earlier flights. Of the organisms obtained in the latest series, sixteen are bacteria and nineteen are molds.

As these collections are made during flights which simultaneously procure air mass data, the possibility of correlating the presence of any particular micro-organism with different types of air masses is apparent.

In table 1 the micro-organisms collected and identified in whole or in part are cited, together with the altitude ranges at which they were collected.

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PLATE 1

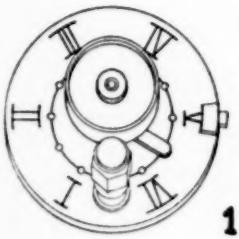
FIG. 1. The lower half of the enclosing case showing the centrally-located hand wheel by which the disc is turned. Between numerals *I* and *V* is the covered suction outlet. The cap is kept on until the collector is installed in the plane. The case is held together by an extension of the central shaft of the hand wheel. This shaft is not visible on this drawing.

FIG. 2. The collecting disc showing the six large collector holes in which the oiled collecting slides are mounted. Between these holes are the six smaller by-pass holes.

FIG. 3. The upper half of the enclosing case. The inlet hole is barely visible in the lower portion of the drawing.

FIG. 4. The assembled apparatus with the collecting orifice opened.

PLATE I



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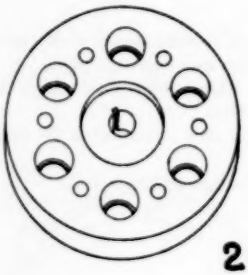

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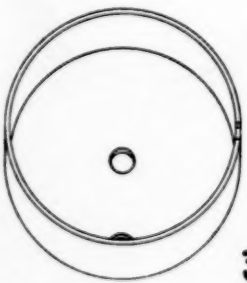

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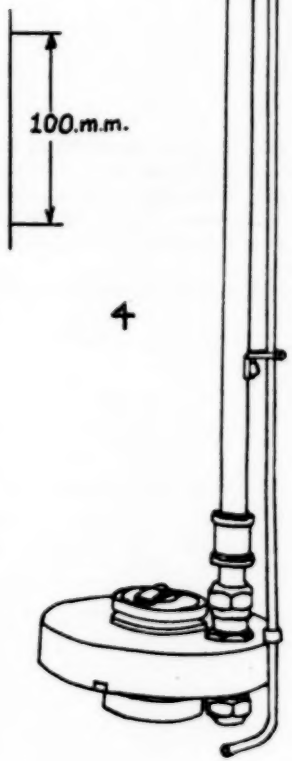


4

PLATE 2

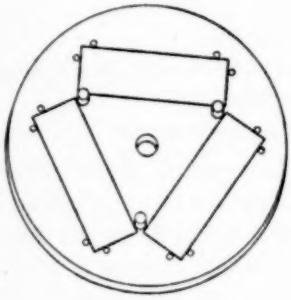
FIGS. 5 AND 7. Brass Plates for applying protective microscope slides to the collector disc preparatory to laboratory examination and photography.

FIG. 6. Enlarged view of a collecting slide and slide holder with which each of the six large holes in the disc (plate 1, fig. 2) is fitted.

FIG. 8. Pedestal plate upon which the protected disc is mounted for examination.

Figs. 5, 7 and 8 are drawn to the same scale as the figures in plate 1.

PLATE II



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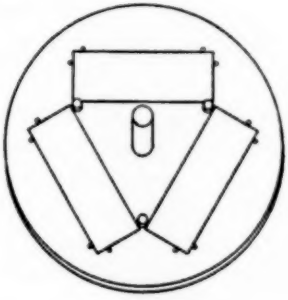

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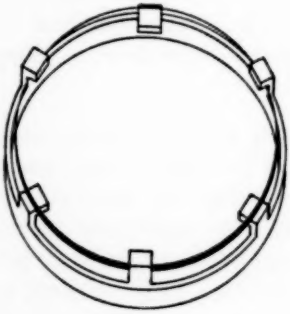

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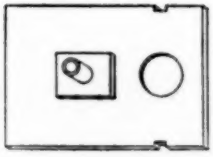

8

COLCHICINE STIMULATION OF YEAST GROWTH FAILS TO REVEAL MITOSIS

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The formation of the yeast bud has generally been believed to be a partitioning of the materials between the mother cell and the bud, rather than involving a mitotic division of the nuclear material. Three investigators have presented evidence for a mitotic division, but the evidence has not been entirely convincing. Colchicine delays mitotic division at the metaphase and has been helpful in the study of cell division in both animal and plant cells. A proper concentration of the drug should delay any mitotic division of yeast, change the rate of growth and permit the observation of division figures. Instead, colchicine stimulates yeast growth and fails to show mitosis.

I

The yeast used is a pure strain of *Saccharomyces cerevisiae* Hansen from a single cell isolated by the writer in 1928 (A. T. C. No. 4360) and was grown at 28°C. in 10 ml. Williams' original medium consisting of sugar 20 grams, $(NH_4)_2SO_4$ 3 grams, KH_2PO_4 2 grams, asparagin 1.5 grams, $CaCl_2$ and $MgSO_4$ each 0.25 gram aq. dest. 1000 ml. The medium was tubed, sterilized and inoculated as described by Richards (1932). Mallinckrodt's U. S. P. colchicine was used in the experiment in preference to Merck's colchicine as the latter was not chloroform free. Two check experiments with the Merck drug gave very nearly the same results so that with yeast either might have been used and without significant differences.

[1] Experiments series 162 and 163 were grown at New Haven, 164 at Woods Hole and 155 and 156 at Buffalo.

The growth of the yeast was measured by counting the number of cells present in 1/250th cu. mm. with an hemocytometer; by means of a photoelectric nephelometer (Richards and Jahn, 1933), improved by using a General Electric Blocking Layer Photoelectric Cell and a Weston Model 600 microammeter with knife edge pointer and mirror; and by centrifuging the cells into calibrated, capillary bottom, centrifuge tubes. More than one criterion of growth is necessary for the analysis of the conditions determining growth (Richards, 1934) and the interrelations of the several criteria will be discussed elsewhere. The metal surface of the Bright Line Hemocytometer permits more rapid filling of the cell by capillarity, thereby lessening the error due to unequal distribution of the yeast. The growth measured by the nephelometer is a function of the number of cells present and their size distribution and is expressed as

$$E = \log I - \log I_0$$

when I is the density of the suspension at the time of measurement and I_0 the density of the suspension at the time of seeding the population.

II

Concentrations of colchicine weighed out and added in the proportion of one to one million parts of medium gave very little less growth than the control populations and concentrations greater than ten times this amount stimulated the yeast growth progressively until a concentration of 1 per cent was reached. Four and one-half per cent concentration, which is near the solubility limit of the drug, gave less stimulation (fig. 1, *A*). In terms of percentage of the control the amounts of stimulation of cell number and nephelometric density were about the same and the agreement of the several determinations is shown by the figure. The average size of the yeast cells grown in the medium and in the medium plus 1 per cent colchicine and the distribution of the sizes of the cells showed no significant differences; as determined by photographing the yeast, projecting the images of the photographs, tracing them and measuring the area of the

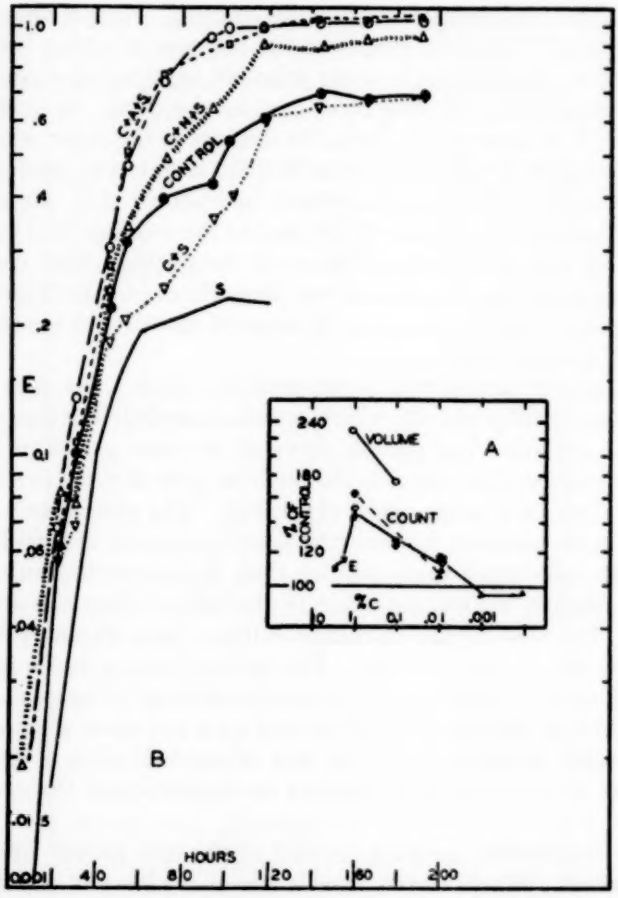

FIG. 1. *A*, THE INCREASED YIELD OF YEAST WITH ADDED COLCHICINE. *B*, THE GROWTH OF YEAST POPULATIONS IN DIFFERENT COMBINATIONS OF NUTRIENTS (*cf.* TEXT)

Control in Williams' medium. *C* = colchicine. *S* = sugar. *A* = asparagin. *N* = $(NH_4)_2SO_4$.

enlarged photographs with a planimeter. Consequently, the proportionally greater volume must be due to inefficient packing of the cells into the capillary tubes by the centrifuge.

The increased yield of yeast, while marked, is not of the order of magnitude to be expected from the presence of a bios, because far greater concentrations of the drug are required for a comparable stimulation. To determine the nature of the stimulation, series 164 was analysed to learn the changes in the sugar, alcohol, and hydrogen ion concentrations at frequent intervals during the growth cycle. Previous experience had shown that the sugar concentration is a measure of the food in the medium used by the cells and that the unfavorableness of the medium from the accumulation of waste products was proportional to the fermentation which could be measured in terms of the alcohol concentration (Richards, 1932).

The growth of the cells in the medium without the drug produced two cycles (fig. 2), which are characteristic for this yeast in this medium. The growth curve of the yeast grown in 1 per cent colchicine in addition to the medium grew directly to a maximum crop in a single cycle of growth. The concentration of sugar in the medium is about the same during the first 60 hours and then decreases more rapidly than in the control cultures. The hydrogen ion concentration is diminished when the drug is added, but that of the colchicine cultures then increases faster than in the control cultures. The alcohol concentration in the colchicine cultures increases to a maximum some six times greater than that of the control cultures and does not show a period of equilibrium associated with the end of the first cycle of growth such as is observed in the alcohol concentration of the control series.

The equilibrium marking the end of the first growth cycle of the control cultures results from a selective killing of the larger buds by the toxic waste products in the medium and a decrease in the available food supply. The addition of food just before the end of the first cycle prevents the retarded growth and the population continues directly to a maximum crop limited by the medium becoming too unfavorable for further increase (Richards, 1934).

As only one cycle occurs when the colchicine is present, the addition of the drug must act either as a food or by making the

medium less unfavorable even though the alcohol and other products of fermentation are proportionally greater. To evaluate the extent of these two possibilities an experimental series was grown in the complete medium, the medium with 1 per cent

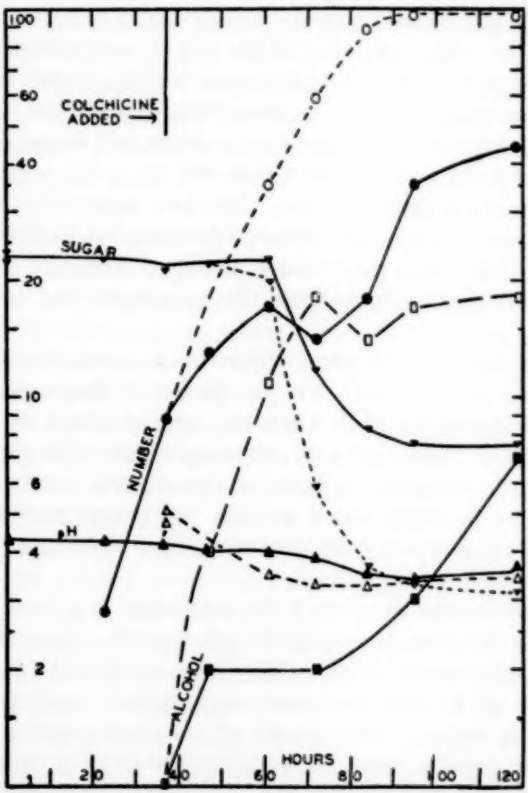

FIG. 2. INCREASED NUMBER OF YEAST PRODUCED BY THE ADDITION OF COLCHICINE AND THE CHANGES WITH TIME OF THE SUGAR, HYDROGEN ION AND ALCOHOL CONCENTRATIONS OF THE MEDIUM WITH AND WITHOUT THE DRUG

colchicine, and in 1 per cent colchicine with the mineral salts and the following combinations of nutrients: sugar, ammonium sulfate, asparagin, asparagin and ammonium sulfate, asparagin and sugar, and ammonium sulfate and sugar, the concentrations being

exactly the same as in the control medium except for the nutrients intentionally omitted.

Without sugar, little growth was observed. With the sugar and the mineral salts an appreciable growth occurred, (fig. 1, *B*). This curve was added from a previous series started with a smaller seeding which accounts for the curve being to the right of the others. The similarity of the slopes, which show the relative rate of growth from the plotting on arithlog paper, show that the rate was essentially the same except for less yield. The addition of 1 per cent colchicine to the sugar and mineral salts increases the yield nearly three times and there is a suggestion of two cycles in the growth curve. The crop attained is the same as that of the control series without the drug but in the complete medium. The yeast may obtain nitrogen from the colchicine, although not as readily as from the asparagine and ammonium sulfate.

The addition of ammonium sulfate to the colchicine and sugar gives a still greater yield, but the period of decreasing rate of growth occurs earlier than when the drug is added to the complete medium. The colchicine, asparagin and sugar gives a crop similar to the complete medium, so that the colchicine serves as well without the ammonium sulfate. Asparagin and colchicine without sugar give less yield than with sugar alone and this curve was not plotted.

The experiments show that the colchicine is a food and also buffers the medium, lessening the effect of the increasingly adverse medium on the yeast proliferation so that the population grows directly to the maximum crop without passing through two growth cycles. The period of constant relative rate, or logarithmic growth, lasts longer when the drug is present with an additional source of nitrogen, preferably asparagin, despite the fact that the alcoholic fermentation is greater and the sugar is used up more rapidly.

III

Had the colchicine arrested the division, or bud formation of the yeast as it does with other plants and animals (Nebel, 1937; Allen, 1937) the rate of growth of the populations would have

been less than of the control populations grown without the drug. Even a small retardation would be evident during the period of exponential rate of increase and would appear as a changed slope of the growth curves. The logarithmic growth period continues beyond that of the control cultures when the colchicine is added to the culture medium and the departure from the straight line in the figures plotted on arithlog paper occurs later and less rapidly. The slightly lesser crops obtained with the lower concentrations of the drug differ from the controls by less than the experimental error (4 per cent) and the differences are not significant (fig. 1, *A*).

Samples of yeast were prepared for microscopic examination by killing in Lavdovsky's or Bouins fluids, mordanted in 2.5 per cent iron alum, stained in 1 per cent aqueous hematoxylin, destained in iron alum or in picric acid and mounted with glycerine jelly. Some of the preparations were made in bulk and the cells separated from the various fluids by gentle centrifuging and the rest as cover glass preparations.

None of the stained preparations showed any grouping of the chromatin that might resemble chromosomes. The chromatin granules were irregularly distributed between the mother cell and the bud depending somewhat on the size of the bud. I failed to note any distinguishing difference between the appearance of the colchicine-treated and the control cells. The yeast were examined at different stages during the growth cycle.

Wager (1898), Wager and Peniston (1910) and Guilliermond (1904–1912), among others, failed to find mitosis in their extensive studies. Fuhrman (1906), Swellengreble (1905) and Kater (1927) describe a mitosis of the yeast nucleus with four chromosomes. The first mentioned paper seems not to be entirely clear. The other two papers publish clear descriptions and figures. Unfortunately the preparations of Kater have faded and are not available for examination.

No yeast cell that I have seen has shown any grouping of chromatin that might be interpreted as chromosomes. Feulgen-stained preparations have been studied (1933). This includes examination of my own preparations and some preparations very

carefully stained by Dr. H. W. Beams for this purpose. The ultra-violet photographs of Ter Louw and of Dr. F. F. Lucas do not show chromosomes (personal communications to the author). The preponderance of negative evidence does not answer the question in a satisfying manner, but it is peculiar that only three observers have been able to see mitotic figures if the budding of yeast is mitotic. It was hoped that the colchicine would make possible the demonstration of mitotic division as it has done with such success for other cells. The drug failed to show any effect on cell division although it does stimulate the growth of populations of yeast.

SUMMARY

The colchicine technique was applied to the study of the growth of yeast populations and failed to show any evidence of other than amitotic cell division. Stained preparations of the cells grown in Williams' medium and in the medium plus from 4.5 per cent to 1 part of colchicine to ten million parts of medium showed no differences in cytological structure. One per cent of the drug gave maximum stimulation of the yeast growth. The stimulation is not of the kind given by a bios, but is due rather to the colchicine serving as a food and as a buffer in lessening the adverse effect of the increasingly unfavorable medium on the growth of the yeast populations.

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A SIMPLE METHOD FOR THE STERILE FILTRATION OF SMALL AMOUNTS OF FLUID

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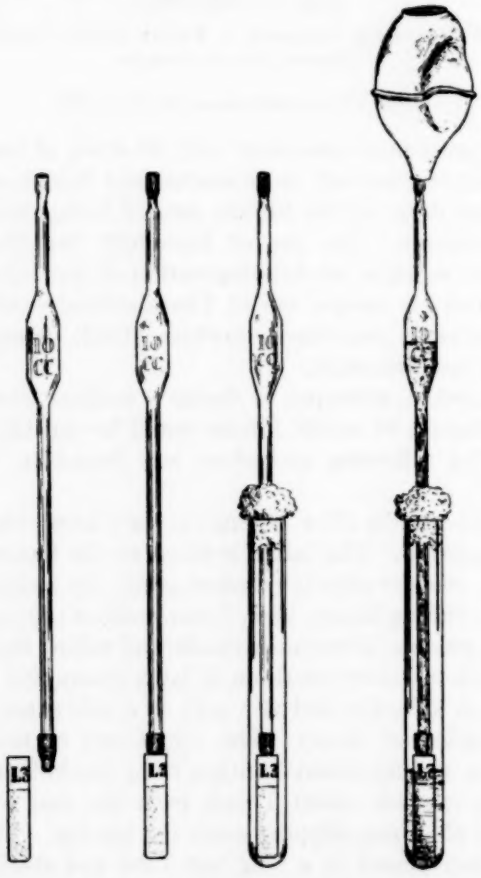
In many procedures associated with the study of bacteriophage the filtration of relatively large amounts of fluid is seldom necessary, a few drops of the filtrate usually being quite sufficient for trial purposes. The use of Berkefeld, Mandler, Seitz or similar filters requires careful preparation of apparatus and even the comparatively simple use of Chamberland candles or Collodion membranes described elsewhere (1933) demands considerable time and attention.

After numerous attempts to devise a method whereby a few cubic centimeters of sterile filtrate could be quickly and easily obtained, the following procedure was found to be entirely satisfactory:

A miniature candle (55 x 10 mm.) of the Chamberland L_3 or L_5 series is employed. The latter is fitted to the tapered end of a 10 or 20 cc. transfer pipette (student grade) by means of a short piece of thin tubing (3 mm. bore, 2 mm. wall) of pure gum rubber. To prevent leakage between the candle and tubing the connection is sealed with a watery emulsion of latex (marketed under various names as adhesive and also sold as a substitute for leather in the repairing of shoes). The upper end of the pipette is shortened to a point about 3 inches from the bulb and a slight constriction is made about 1 inch from the end to prevent a cotton wool plug from slipping down the pipette. The mounted candle is then placed in a long test tube and sterilized in the autoclave.

When required for use, the candle is immersed in the fluid to

be filtered approximately half way to the glazed neck. Suction is applied to the end of the pipette either by fitting over it a compressed india-rubber bulb or preferably by connecting it to a vacuum pump. When the latter is employed, about 10 cc. of sterile filtrate can be obtained in less than five minutes, even with heavy bacterial suspensions.



After sufficient filtrate has appeared in the bulb of the pipette, the latter is disconnected from the pump, the cotton plug re-

moved, the end flamed and the filtrate withdrawn with a Pasteur pipette. The entire process requires only five or ten minutes and a large number of test filtrates can be obtained in a short time.

To keep the candles in a satisfactory condition for prolonged and repeated use, the following simple procedure is recommended:

Using the same Pasteur pipette that was employed for collecting the filtrate, fill the volumetric pipette with distilled water and allow the latter to flow back through the candle by gravity, thus removing any material adhering to the outside wall of the candle. After sterilization in the autoclave, the candle is disconnected from the pipette, dried and burned in a muffle furnace at dull red heat for 1½ hours. Filter candles thus treated may be used repeatedly and will remain in a satisfactory condition for an indefinite period.

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NUTRITION OF THE PROPIONIC ACID BACTERIA[1]

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Received for publication March 11, 1938

Wood, Tatum and Peterson (1937) and Tatum, Wood and Peterson (1936a) have shown that an ether-extractable acidic factor found in aqueous extract of yeast is essential for growth and fermentation of propionic acid bacteria in a synthetic medium containing ammonium sulfate as the source of nitrogen. Although growth was obtained with this combination, the organisms generally failed to multiply after the third or fourth transfer. The addition of vitamin B_1 and hydrolyzed casein made repeated transfer possible with consistent results. Vitamin B_1 stimulated growth, particularly in the presence of amino acids. Certain propionic acid bacteria were found to grow with equal vigor with or without the addition of vitamin B_1 and may be able to synthesize this vitamin.

These investigations indicated that amino acids were essential for vigorous and continuous growth in the supplemented synthetic medium. The present study was made to determine which of the amino acids are important in the general nutrition of propionic acid bacteria. It has been found that amino acids are not essential for growth.

EXPERIMENTAL

Cultures

Each of the seven species used was checked for purity by fermentation of the differential sugars in the classification of Werkman and Brown (1933). The following cultures were used:

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Propionibacterium rubrum 9W; *P. peterssonii* 11W; *P. thönii* 23W, 39W; *P. zeae* 35W; *P. pentosaceum* 49W; *P. shermanii* 52W; and *P. freudenreichii* 53W. Cultures 35W, 49W and 53W are transplants of cultures 56, 11 and 33 respectively, as referred to in the previous studies by Tatum *et al.* (1936a) and Wood *et al.* (1937).

Medium and analytical methods

The media developed in previous work were used throughout this investigation. The basal medium contained: glucose, 1 per cent; sodium acetate, 0.6 per cent; ammonium sulfate, 0.3 per cent; and Speakman's salts in half concentration. The growth stimulants were added in various combinations and in the following concentrations: ether extract of 0.3 gram of Difco yeast extract; 0.1 gamma of crystalline vitamin B_1 (Merck's); 10 gamma of crystalline riboflavin (Borden) per 10 cc. of medium, unless the concentration is otherwise designated. The amino acids were used in a concentration of 0.02 per cent on the basis of the naturally occurring isomer with the exception of cystine and tyrosine which were used in a concentration of 0.01 per cent. The following amino acids were used: glycine, dl-alanine, dl-valine, dl-isoleucine, dl-leucine, dl-phenylalanine, l-tyrosine, l-cystine, dl-methionine, l-tryptophane, l-proline, l-hydroxyproline, l-aspartic acid, d-glutamic acid, d-arginine, dl-lysine and l-histidine. Hydrolyzed casein was used in 0.15 per cent concentration. Ammonium sulfate was added to all media containing amino acids, as it was our purpose to determine the influence of amino acids in the presence of ammonium sulfate. The constituents of the media were adjusted to pH 7.0, combined, diluted to volume with distilled water, and sterilized at 15 pounds pressure for 20 minutes. Five cubic centimeters of medium were used in test tubes 1 cm. in diameter. Incubation was aerobic at 30°C. for 4 or 5 days. The inocula obtained from yeast extract medium were centrifuged and resuspended in an equivalent volume of water; cells grown in supplemented synthetic medium with ammonium sulfate as a nitrogen source were not centrifuged. One drop of inoculum was used per tube

unless otherwise stated. Fermentation was followed by direct titration of the acid formed, with bromothymol blue as an indicator.

Amino acid requirements

The results (table 1) show clearly that amino acids have an important function. Four cultures having no amino acids gave only slight growth and no acid on the second transfer; on the third transfer there was no visible growth in four days. Culture 35W grew more vigorously than the other cultures; it was not until the fifth transfer that all visible growth disappeared. The addition of amino acids stimulated growth of each of the cultures. There was a luxuriant development of cells and consistent acid production throughout five transfers. Implications at this time were that one or more of the seventeen amino acids was essential and that growth was not possible with ammonium sulfate after material included in the inoculum was depleted by serial transfer. In an attempt to determine which were necessary, the seventeen purified amino acids were separated into the six groups shown in table 2. Two cultures were used in the test. Experiments with culture 49W were run independently; transfers were made with culture 52W from the first set of tubes (c) to a second set (d) in which the amino acids were one-half as concentrated. The first tests were made by eliminating singly each of the six groups of amino acids to determine whether any one group contained essential amino acids (numbers 1 to 7). No single group of amino acids was essential. With culture 49W the elimination of groups IV and V (numbers 5 and 6) caused no decrease in growth compared with a medium containing all six groups (number 1), indicating that groups I, II, III and VI were the more important for this culture. Culture 52W showed some decrease in growth on elimination of each single group from the six, but groups I, II and III seemed the more important, particularly in series (d). The combination of groups I, II, III and VI were tested next (number 8). Culture 49W grew practically as well as with the complete set of amino acids (number 1) or hydrolyzed casein (number 25). Culture 52W did not give as good growth

on this combination as on the seventeen amino acids or hydrolyzed casein but again (in series (d)) groups I, II and III (numbers 9, 10, 11) seemed important. Growth was retarded by the removal of any one of these groups. Where two groups were used (numbers 13 to 17) the growth was usually somewhat weaker than with three, and with a single group growth was retarded even more. Cystine (number 23) seemed to have a favorable influence but growth on a second transfer from this tube was weak. It is apparent that clear cut differentiation of the

TABLE 1

Comparison of ammonium sulfate and purified amino acids as a source of nitrogen for propionic acid bacteria*

Medium contained B_1 and ether extract of yeast extract

NUMBER OF TRANSFERS	CULTURE 11W		CULTURE 35W		CULTURE 39W		CULTURE 52W		CULTURE 53W	
	Amino acids not added	Amino acids added	Amino acids not added	Amino acids added	Amino acids not added	Amino acids added	Amino acids not added	Amino acids added	Amino acids not added	Amino acids added
	Cubic centimeters of 0.1 N acid per 10 cc. of medium									
1	1.6	2.1	3.3	4.0	0.2	2.9	1.1	4.2	1.1	4.0
2	0.0	3.0	1.7	4.1	0.0	2.6	0.0	5.1	0.0	3.8
3		2.6	1.2	3.5		2.8		4.5		3.3
4		2.7	0.0	4.5		2.5		5.1		4.3
5		3.1		4.7		3.0		4.5		4.1

4-day incubation. Initial inoculum was obtained from yeast extract glucose medium.

* Mixture of seventeen amino acids, 0.02 per cent of each except cystine and tyrosine 0.01 per cent, calculated on the basis of the natural isomer.

important amino acids is not possible, at least under the conditions of these experiments. Possibly by direct determination of cell weight with a short incubation period, the more important amino acids could be ascertained. However, it seemed that uncontrolled factors other than the methods of analysis were influencing our results. Subsequent data in this paper show that these cultures readily acquire the ability to synthesize amino acids, and can utilize ammonium sulfate as the source of nitrogen if properly "trained" in the sense of Knight (1936) who defines the term as "the derivation of cultures having simple nutrient

TABLE 2

Comparison of amino acid mixtures as a source of nitrogen for the propionic acid bacteria

Medium contained ether extract of yeast extract and B_1

NUMBER		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
I. Glutamic, leucine, histidine, methionine		+		+	+	+	+	+	+		+	+	+	+		+			+							Hydrolyzed casein
II. Glycine, alanine, valine		+	+		+	+	+	+	+	+		+	+	+	+		+			+						
III. Isoleucine, aspartic, lysine, arginine		+	+	+		+	+	+	+	+	+		+		+	+		+			+					
IV. Proline, hydroxproline, phenylalanine, tryptophane		+	+	+	+		+	+														+				
V. Tyrosine		+	+	+	+	+		+															+			
VI. Cystine		+	+	+	+	+	+		+	+	+	+					+	+						+		
Number of groups present		6	5						4	3				2					1						0	
cc. of 0.1 N acid produced per 10 cc. of medium	Culture 49W a*	5.0	4.1	4.8	4.1	5.1	5.3	3.7	4.9		4.8	3.2	4.5	3.6											2.0	5.0
	Culture 49W b*	4.8		4.3	3.7	4.8	4.9		4.6		4.6					3.4		4.0							1.8	5.0
	Culture 52W c*	4.9	4.5	3.7	4.0	4.4	4.5	4.4	4.6	3.7	3.1	3.1	2.6		3.2	3.2	2.0	2.8							0.3	4.6
	Culture 52W d*	4.0	3.4	3.0	2.7	3.6	3.6	3.5	3.6	2.4	2.5	2.7	3.6		2.6	2.1	1.8	2.4	0.7	1.9	1.5	0.5	0.3	2.8	0.4	4.0

4-day incubation.

* a, b and c tubes contained 0.02 per cent of each of the amino acids tested except tyrosine and cystine (0.01 per cent); d tubes contained 0.01 per cent each of the amino acids tested except tyrosine and cystine (0.005 per cent). Inoculum for a and b tubes obtained from supplemented synthetic medium. c tubes' inoculum obtained from yeast extract glucose medium. d tubes inoculated from corresponding c tubes except number 18 through 23 were inoculated with number 24.

requirements from cultures with a complex nutrition." It seems probable that in the presence of a few amino acids the ability to dispense with other amino acids is acquired readily. This ability may account for the lack of specificity for any particular amino acid as illustrated in table 2. Gladstone (1937) has made similar observations with *Staphylococcus* showing that "training" to synthesize one amino acid may enhance the ability to synthesize other amino acids. The poor growth on ammonium sulfate medium may result from the difficulty of "training" in this medium. Apparently, in the absence of all amino acids the bacteria only slowly acquire an ability to synthesize those amino acids that are essential. The differences in growth noted in table 2 on the elimination of certain groups of amino acids are probably a measure of the difficulty of synthesizing or dispensing with, a certain amino acid.

Stimulation of growth by riboflavin

Wood, Andersen and Werkman (1937) reported the stimulating effect of riboflavin on propionic acid bacteria. Further results are presented in tables 3, 4 and 6. Table 3 shows the acid production during serial transfer of three cultures in ammonium sulfate medium, with and without the addition of riboflavin. It is apparent that riboflavin had a stimulating effect on the growth of each culture. The growth of 35W and 49W was consistently vigorous throughout six transfers when flavin was added but decreased and was very weak at the conclusion of the experiment in its absence. Culture 52W failed to grow after the fourth transfer. The stimulating influence of the flavin was evident with this culture particularly in the early stages of growth. It served to shorten the lag phase and turbidity was evident much earlier. Cultures 9W, 11W, 23W, 39W and 53W failed to grow in either medium. It was necessary to "train" these cultures in order to obtain even moderate growth in ammonium sulfate medium containing flavin. These organisms apparently do not acquire the ability to synthesize amino acids as readily as others. On the other hand they are able to synthesize or dispense with flavin in amino acid medium for this

substance was not necessary for vigorous growth in the presence of amino acids (table 1). The effect of different concentrations of riboflavin on acid production by propionic acid bacteria in ammonium sulfate medium is shown in table 4. Five-tenths

TABLE 3

Effect of riboflavin on acid production by propionic acid bacteria

Medium contained ether extract of yeast extract, B_1 and no amino acids

CULTURE*	FLAVIN ADDED TO 10 CC. OF MEDIUM	NUMBER OF TRANSFERS					
		1	2	3	4	5	6
		Cubic centimeters of 0.1 N acid per 10 cc. of medium					
	gamma						
35W	None	2.3	2.0	2.4	0.9	1.2	0.5
	10	3.7	3.5	4.5	3.2	4.0	4.2
49W	None	3.1	2.2	2.8	1.4	1.0	0.2
	10	3.6	3.6	4.4	3.6	4.0	3.7
52W	None	3.3	2.7	3.1	0.1	0.0	
	10	3.6	3.6	4.0	0.6	0.0	

5-day incubation. Initial inoculum was obtained from yeast extract medium.
* Cultures 9W, 11W, 23W, 39W, 53W failed to grow in this experiment.

TABLE 4

Effect of concentration of riboflavin on acid production by propionic acid bacteria

Medium contained ether extract of yeast extract, B_1 and no amino acids

CULTURE NUMBER	GAMMA OF FLAVIN ADDED PER 10 CC. OF MEDIUM				
	None	0.5	5.0	10.0	20.0
	Cubic centimeters of 0.1 N acid per 10 cc. of medium				
35W	1.3	2.6	3.2	4.0	4.1
49W	2.2	2.7	3.6	4.4	4.4
52W	2.9	3.5	3.6	3.6	4.1

5-day incubation. Inoculum was obtained from second transfer on the supplemented synthetic medium containing no flavin or amino acids.

gamma of riboflavin per 10 cc. had a distinct effect. The maximum influence was reached at 10 gamma per 10 cc. Snell, Strong and Peterson (1937) observed a stimulation of lactic acid bacteria by 0.05 gamma per 10 cc. and a maximum at 0.5

gamma per 10 cc. Orla-Jensen, Otte and Snog-Kjaer (1936) first observed the stimulating effect of riboflavin on lactic acid bacteria. Lava, Ross and Blanchard (1936) suggested that flavin or B_2 might be a stimulant for propionic acid bacteria but were unable to prove this because of the impurity of their preparation. They fractionated yeast extract and found the fraction that contained B_2 was the most active. In the present investigation crystalline riboflavin was used and the effect is definitely shown to be produced by the flavin.

"Training" and the effect of size of inoculum

The same medium was inoculated with 1, 2 and 5 drops of the suspension per 5 cc. The cells for the inocula were obtained by centrifugation from yeast-extract glucose medium and were suspended in distilled water. Each series was carried through four serial transfers. There is evidence of adaptation to the ammonium sulfate medium, particularly by cultures 9W, 11W and 23W. These cultures show increased acidity with increasing inocula in each of the four transfers. With a given size of inoculum the acid formed increased with the number of transfers up to the fourth when the acid was about the same as in the third. Increased growth during serial transfer indicated that the bacteria were becoming acclimated. Apparently the ability to synthesize amino acids increased during each transfer. The growth was uniform on serial transfer when amino acids were supplied (table 1). Presumably this adaptation or "training" occurs more readily when a large inoculum is used. It does not seem probable that the increased growth is due directly to the material from the yeast extract which is carried over; if this were true and adaptation did not occur, growth would decrease rather than increase as the material was depleted by serial transfer. It is probable that the "carry over" facilitates growth on the first transfer and this speeds up adaptation by the organism. Cultures 39W, 52W and 53W showed little difference with size of the inoculum or number of transfers. Previously 9W, 11W, 23W, 39W and 53W failed to grow with one drop of inoculum in this medium (table 3). No explanation can be given for the

change. It illustrates an alteration in the nutritional requirements of bacteria; in this case the conditions influencing the change were not controlled.

Dispensability of ether extract of yeast extract, vitamin B_1, and riboflavin in ammonium sulfate medium

Different combinations of the three factors were tested with eight cultures in serial transfer (table 6). There was considerable variation in the response of different cultures, which may be due in part to the nutritional state of the organism, i.e., its power to synthesize essential substances at the time studied. Cultures 35W and 49W have been the most consistent in their ability to grow in ammonium sulfate medium, but 52W and 53W grew more vigorously after they were "trained" to this medium. Cultures 9W, 11W, 23W and 39W experienced more difficulty in growth. A different sample of ether extract of yeast extract was used and it was not as effective as the sample used in the experiment of table 5. Briefly, the results in table 6 show that the ether extract of yeast extract is sufficient stimulant by itself to allow growth of 35W, 49W, 52W and 53W. The latter two cultures showed no further stimulation by additional factors. They may be able to synthesize or dispense with these factors. Ether extract of yeast extract was essential for all cultures, as shown by the absence of growth in medium containing only B_1 and flavin. The combination of the extract of yeast and flavin was no more effective than the yeast factor alone. B_1 shows a definite stimulation in the presence of the yeast factor with cultures 23W, 35W, 39W and 49W. All three factors were influential in promoting growth of 9W, 11W, 23W and 39W. This stimulation by flavin supports the previous results shown in table 3 and provides additional evidence that flavin stimulates growth of propionic acid bacteria. Cultures 35W, 49W and 52W apparently acquired the ability to synthesize flavin or dispense with it during growth in ammonium sulfate medium. Conditions controlling the development of this ability are not fully understood, inasmuch as the stimulating effect of flavin could not be uni-

TABLE 5

Effect of size of inoculum on acid production by propionic acid bacteria

Medium contained ether extract of yeast extract, B_1, riboflavin and no amino acids

SIZE OF INOCULUM	NUMBER OF TRANSFERS											
	1	2	3	4	1	2	3	4	1	2	3	4
	Cubic centimeters of 0.1 N acid per 10 cc. of medium											
	9W				11W				23W			
drops												
1	0.3	1.0	1.4	1.3	0.7	0.9	1.0	1.0	0.3	0.3	1.6	1.6
2	0.7	1.4	1.7	1.9	0.8	1.0	1.6	1.5	0.5	1.1	2.7	2.7
5	1.1	1.7	2.7	2.3	1.1	1.4	2.0	2.1	0.7	1.6	3.2	2.8
	39W				52W				53W			
1	1.2	1.2	1.2	1.2	2.8	3.0	3.3	2.8	2.9	2.8	2.9	2.9
2	1.6	1.5	1.7	1.6	2.9	3.2	3.4	3.2	3.2	2.8	2.9	3.3
5	1.6	1.6	1.4	1.3	3.3	3.3	3.5	3.4	3.3	2.9	2.7	3.2

5-day incubation except 7-day with 23W. Inoculum obtained from yeast extract glucose medium.

TABLE 6

Effect of ether extract of yeast extract, B_1 and riboflavin on acid production by propionic acid bacteria

MATERIAL ADDED TO BASAL MEDIUM*	NUMBER OF TRANSFERS																			
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
	Cubic centimeters of 0.1 N acid per 10 cc. of medium																			
	35W					49W					52W					53W				
Y	2.4	1.7	2.2	2.4	†	2.3	2.2	1.6	1.9	1.9	4.2	4.5	3.4	4.4		3.4	1.9	3.9	3.5	†
B_1F	0.1					0.1					4.1	0.1				3.9	0.1	0.1		
YF	2.4	1.4	0.3	2.9	†	2.7	2.3	0.9	2.1	0.8	3.8	3.4	3.3	†		3.9	3.5	3.3	†	
YB_1	4.2	2.4	3.5	3.4	†	4.5	4.5	4.7	4.6	4.5	4.4	4.0	3.1	†		3.5	3.4	3.3	†	
YB_1F	4.5	2.2	3.4	3.5	†	4.6	4.6	4.4	5.0	4.7	3.9	3.3	3.1	†		3.9	3.7	3.3	†	
	9W					11W					23W					39W				
Y	0.7	0.2	0.1			0.5	0.1	0.1			1.0	0.3	0.2			0.5	0.1	0.1		
B_1F	0.9	0.1				0.5	0.1				1.0	0.1				0.8	0.1			
YF	0.7	0.3	0.2	0.2		0.5	0.3	0.2	0.2		0.9	0.3	0.2	0.2		0.7	0.1			
YB_1	0.8	0.4	0.3	0.3	0.2	0.5	0.3	0.3	0.2	0.1	1.0	0.9	0.4	0.7	0.5	0.8	0.7	0.5	0.8	1.4
YB_1F	0.9	1.1	1.0	1.4	1.3	0.7	0.9	0.7	1.3	1.3	1.3	1.1	0.9	1.3	1.0	0.9	1.0	1.0	1.1	1.4

7-day incubation except 5-day with 35W and 49W. Inoculum obtained from supplemented synthetic medium.

* B_1 = 0.1 gamma vitamin B_1, F = 6.0 gamma riboflavin, Y = ether extract of 0.3 grams of yeast extract, per 10 cc. medium.

† Not transferred.

formly demonstrated with cultures taken directly from the stock cultures in yeast extract medium.

Another instance of change in nutritional requirements may be cited in which culture 49W was "trained" to grow as well in the absence of vitamin B_1, as in its presence. This adaptation occurred after continuous serial transfer in ammonium sulfate medium containing only the yeast factor as a stimulant. The titrations following the five shown in table 6 were 6th = 1.0, 7th = 2.6, 8th = 2.8, 9th = 3.0, 10th = 3.6, 11th = 4.8, 12th = 4.8. Apparently the culture gradually acquired the ability to dispense with vitamin B_1.

It has been known that the nutritional requirements of bacteria can be changed by alteration of the substrate. The literature has been reviewed by Knight (1936). In the present work we have been able to "train" cultures to dispense with amino acids, flavin and vitamin B_1.

Replacement of yeast factor by other stimulants

Knight (1937) has shown that nicotinic acid and vitamin B_1 are able to replace the *Staphylococcus* factor and in addition Richardson (1936) has shown that uracil is necessary for anaerobic growth of certain staphylococci. Mueller and Cohen (1937) have found in the case of the diphtheria bacillus that beta alanine, nicotinic acid and pimelic acid are able to replace almost completely the growth factor obtained from liver concentrate. Combination of these factors together with pantothentic acid, kindly furnished by Prof. R. J. Williams, were not able to replace the yeast factor when used in hydrolyzed casein medium containing riboflavin and vitamin B_1. Three concentrations were tested as shown below with cultures 35W and 49W. Quantities are expressed in gamma per cubic centimeter of medium.

	1	2	3
Pantothentic acid	0.02	0.2	2.0
Uracil	0.3	5.0	30.0
Nicotinic acid	0.1	2.0	10.0
Pimelic acid	0.1	2.0	10.0
β alanine	0.1	2.0	10.0

There was some growth on the first transfer but none in subsequent transfers. It is probable that some of these factors are active stimulants for the propionic acid bacteria but the absence of one of the essential factors found in yeast extract prevents growth.

DISCUSSION

Although Tatum, Wood and Peterson (1936b) and Fromageot and Laroux (1936) found that the propionic acid bacteria are able to utilize ammoniacal nitrogen, the present investigation is the first in which these bacteria have been grown during numerous transfers in ammonium sulfate medium with vigorous growth comparable to that obtained in the presence of amino acids. Previously Wood, Tatum and Peterson (1937) had observed inconsistent growth in ammonium sulfate medium during serial transfer. The factor obtained from polenta by Fromageot and Laroux probably is not free of amino acids. The yeast factor is believed to be free of all amino acids. It is obtained by extraction with ether and the material used for this extraction is made strongly acid (pH 2.0). Under these conditions amino acids are not extracted as shown by the ninhydrin reaction. The extract does contain nitrogen, however.

The question of whether or not propionic acid bacteria which are "trained" to dispense with B_1 and lactoflavin acquire the ability to synthesize these compounds cannot be answered at present. It may be that the organisms divert their metabolism so that these compounds are not necessary. As yet we have not tested the medium of "trained" cultures to determine whether vitamin B_1 and riboflavin are formed.

The physiological function of these factors is a subject of interest about which we know little at present. The procedure applied by Lwoff and Lwoff (1937) in the study of the "V" factor with *Haemophilus influenzae* showing it to be a codehydrogenase is instructive and might be useful in a similar study with these organisms. Fromageot and Chaix (1937) have found that compounds of sulfur are important in influencing the critical number of bacteria which are able to activate glucose breakdown. The relation of this factor to those considered here is not clear.

SUMMARY

It has been found that:

1. Amino acids are beneficial but not essential to the propionic acid bacteria. Certain cultures grow with difficulty in their absence, others thrive.

2. Riboflavin (0.05 gamma per cubic centimeter) stimulates growth in ammonium sulfate medium but can be dispensed with by most cultures.

3. Vitamin B_1 is an effective stimulant in ammonium sulfate medium but some cultures can be "trained" to grow vigorously without it.

4. Ether extract of yeast extract is essential for all cultures. It is not replaced by a mixture of nicotinic acid, vitamin B_1, pimelic acid, uracil, beta alanine and pantothentic acid.

5. The nutritional requirements of the propionic acid bacteria are variable, probably depending upon the ability of the culture to synthesize essential substances at the time tested.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

OHIO BRANCH

OHIO STATE UNIVERSITY, COLUMBUS, OHIO, APRIL 30, 1938

STUDIES ON THE MODE OF ACTION OF SULFANILAMIDE. *Robert Finkelstein and Jorgen M. Birkeland*, Department of Bacteriology, Ohio State University, Columbus.

In an effort to explain the mode of action of sulfanilamide and Prontosil in the therapy of hemolytic streptococcus infections, studies were made to determine whether the drugs might act *in vitro* as artificial opsonins. Mixtures of fresh guinea pig leucocytes and hemolytic streptococci were treated with varying amounts of the drugs and incubated for thirty minutes. It was found that in concentrations of from 1:50,000 to 1:100,000 these drugs enhanced phagocytosis. No increased phagocytosis occurred in the absence of fresh serum. When either the leucocytes or the bacteria were treated with the drugs and then washed before mixing with the other no increased phagocytosis was observed. These drugs did not appear to enhance the phagocytosis of *Eberthella typhosa*, *Staphylococcus aureus*, *Brucella abortus* and several types of pneumococci.

PREPARATION OF COLLOIDAL GOLD SOLUTION. *R. D. Scott and Goldie de Long*, Ohio Department of Health Laboratory, Columbus.

In preparing colloidal gold solution, we use the method of Miller and his coworkers (oxalic acid-formaldehyde reduction), except that the reagents are added successively at 50–60°C. instead of at separate temperature stages, and the heating is carried to boiling instead of to 90°C.

The success or failure of preparation is considered largely due to the technic of the formaldehyde reduction which is presented in detail.

No adjustment in reaction is made after preparation; only the behavior with salt solution and with known paretic and normal spinal fluids is noted. Sols of satisfactory appearance thus prepared are, almost without exception, found satisfactory for use in the Lange's test.

DEMONSTRATION OF AN APPARATUS FOR PRESERVING CULTURES IN DEHYDRATED FORM. *George E. Rockwell*, Cincinnati, Ohio.

VITAMIN REQUIREMENTS OF STAPHYLOCOCCUS AUREUS. *Maurice Landy*. Research Division, S.M.A. Corporation, Cleveland, and the Department of Bacteriology, Ohio State University, Columbus.

The experimental work was directed chiefly toward the titration of nicotinic acid and thiamine as growth requirements of *Staphylococcus aureus*, the growth effect of compounds related to nicotinic acid, the testing of 212 strains of *S. aureus* for growth in a completely

synthetic medium, and the significance of bios in the nutrition of this microorganism.

Thiamine and nicotinic acid (or amide) are essential in the nutrition of *S. aureus*, no growth occurring in the amino-acid glucose medium alone, or with the addition of thiamine or nicotinic acid separately. Certain compounds related to nicotinic acid and nicotinic amide cannot be utilized by *S. aureus*. To date, 208 typical strains of *S. aureus* have been grown in an amino-acid glucose medium plus nicotinic acid and thiamine.

Vitamin B_6 and vitamin K show no growth stimulating effect when used under the conditions of our test. Bios is not indispensable in the nutrition of *S. aureus*, but the growth stimulating effect of nicotinic acid and thiamine can be considerably increased by its addition.

THE RÔLE OF THE "X" FACTOR AS A REQUIREMENT FOR GROWTH OF HEMOPHILUS INFLUENZAE. *Thomas L. Snyder and R. H. Broh-Kahn.*[1] Department of Bacteriology, College of Medicine, University of Cincinnati, and the Institute for Medical Research, The Jewish Hospital, Cincinnati.

It is generally acknowledged that *Hemophilus influenzae* requires for growth the presence of the so-called "X" and "V" factors. As the result of an extensive investigation into the nature of these factors, Lwoff has demonstrated not only that protohemin is the "X" factor and Coenzyme I or II the "V" factor, but also has concluded that the function of "X" is to supply a respiratory system to the organism which lacks such a mechanism. If the sole function of the "X" factor were to supply a system for oxygen consumption, it should be possible to replace the "X" factor by a reversible oxidation-reduction system of such potential that it can serve as a respiratory catalyst.

Accordingly, attempts were made to grow *H. influenzae* in a medium containing "V" factor with various oxidation-reduction indicators. Cysteine was added to destroy any hydrogen peroxide accumulating as a result of air oxidation of the leuco dye. In the absence of "X", it was found that growth occurred in the presence of the "V" factor plus either a dye and cysteine, or cysteine alone. As a result of this and other findings, it is concluded that the "X" factor functions by supplying catalase to the organism. The theoretical and practical implications are discussed.

[1] Eli Lilly and Company Research Fellow.

RABIES AS A PROBLEM IN PUBLIC HEALTH. *M. C. Hanson*, Department of Health, Toledo.

"CANNIBALISM" AMONG BACTERIA. *Edward A. Steinhaus and Jorgen M. Birkeland*, Department of Bacteriology, Ohio State University, Columbus.

Plate counts of from 5 to 30 million bacteria per cc. were obtained from nutrient broth cultures of a number of common saprophytic bacteria after a year and a half incubation. In an effort to determine whether the bacterial cells themselves were serving as a source of nutriment in such cultures, media were prepared in which the only known source of food was bacterial cells. Solid media were made from heat-killed cells of *Sarcina lutea*, *Serratia marcescens*, *Staphylococcus aureus*, as well as the whole and the disrupted cells of *Escherichia coli*. These

media were inoculated with 21 different bacteria. In general, the proteolytic organisms grew best on the various media. Definite zones of digestion were evident. Of the 21 bacteria tested, best growth was obtained with *S. lutea, S. marcescens, Bacillus subtilis, Bacillus mycoides, Bacillus anthracis.* Demonstrable but not normal growth was obtained with *E. coli* inoculated on the medium containing *E. coli* cells. The one strain of *S. aureus* used did not support good growth of any of the bacteria tested. *S. marcescens* produced no pigment when grown on cells of *E. coli*, although it did when grown on the media containing other bacterial cells. *S. marcescens* and *S. lutea*, when inoculated into sterile suspensions of the homologous species, gave counts almost as high as in nutrient broth, thus supporting the results obtained on plates of the cell media.

ANAPHYLACTIC SHOCK PREVENTED BY KETENIZING SERUM. *J. T. Tamura and M. J. Boyd*, College of Medicine, University of Cincinnati, Cincinnati.

Ketene was produced by the thermal decomposition of acetone vapors. In general, the reactions of ketene are addition reactions with the amino groups of proteins at neutrality, which enables one to acetylate proteins, properly buffered, without denaturation.

Buffered anti-Brucella horse serum was treated with ketene for various lengths of time. Guinea pigs sensitized to the original anti-serum received intracardially a shocking dose of ketenized anti-serum. Sensitized control animals received the original anti-serum by the same route. The control animals went into shock and died, but guinea pigs receiving anti-serum ketenized for 35 minutes or more showed no anaphylactic syndrome or very mild signs of shock. The ketenized anti-serum retained the major part of its agglutinin antibody.

THE TREND OF SYPHILIS IN CLEVELAND. *James A. Doull and Huldah Bancroft*, Department of Hygiene and Bacteriology, Western Reserve University, Cleveland.

Between 1921 and 1935, 27,976 women were given Wassermann tests in the Prenatal Clinics of Maternity Hospital of Cleveland. The six to eight clinics served nearly the same area from year to year and were fairly wide-spread over the city. First admissions were analyzed separately from re-admissions, as persons found positive on first admission were referred for treatment. For first admissions, percentages positive for white women range from 2.2 to 4.1; for colored women 7.6 to 15.6. The total admissions show in every case a somewhat lower percentage of positives than do re-admissions. For white women there is evidence of a downward trend since 1925. For colored women there is a definite upward trend.

MULTIPLICATION OF VACCINIA VIRUS IN DEEP COLUMNS OF MAITLAND MEDIUM, *Margaret S. Coates and R. L. Thompson*, Department of Hygiene and Bacteriology, Western Reserve University, Cleveland.

By carefully controlling the tissue-fluid ratio, growth of vaccinia virus was obtained in columns of Maitland medium with a depth of over 45 mm. The New York City Board of Health strain of virus was carried through 25 serial transfers in deep cultures without loss in virulence.

PROPAGATION OF THE HUMAN INFLUENZA VIRUS IN THE GUINEA PIG

FETUS.[1] *Oram C. Woolpert, Fred W. Gallagher, Leona Davis, and N. Paul Hudson,* Department of Bacteriology, Ohio State University, Columbus.

The P.R. 8 strain of human influenza virus was inoculated intracerebrally into fetal guinea pigs *in utero*. It was found to multiply and disseminate widely in fetal tissues, attaining high titers particularly in lungs, liver, and placenta. Forty-eight hours proved to be a favorable incubation period. By subinoculating with lung tissue, two series of passages through fetal guinea pigs have been accomplished, one of 10 transfers, the other of 16; the latter is still in progress. The identity and virulence of the virus were maintained during passage, as shown by appropriate tests in mice. It was found that when the virus titer of passage material had been greatly reduced by storage, or otherwise, it could be quickly restored by further passage. It is concluded that the fetal guinea pig is a favorable animal for experimentation with this virus and that large quantities of bacteriologically sterile virus can be made available by the technic described.

ADSORPTION OF POLIOMYELITIS VIRUS BY CHOLESTEROL, ERGOSTEROL, AND OTHER STEROL PRODUCTS. *J. A. Toomey and W. S. Takacs,* Department of Pediatrics, Western Reserve University, Cleveland.

These workers previously demonstrated that cholesterol adsorbs poliomyelitis virus. They then took related products, ergosterol and primary crystalline vitamin D, and were able to demonstrate that these also either adsorbed or inactivated poliomyelitis virus in the same manner as did cholesterol. Their method was to add 10 per cent ergosterol or primary vitamin D to a 1 per cent suspension of virulent virus, grind the combination in a ball mill, and inject the supernatant fluid left after centrifugation into *Macacus rhesus* monkeys. They ground the same amount of virus in physiological saline and injected the suspension into control animals.

PATHOLOGY OF THE HUMAN INFLUENZA VIRUS IN THE GUINEA PIG FETUS. (By Title) *N. Paul Hudson, Fred W. Gallagher, and F. S. Markham,* Department of Bacteriology, Ohio State University, Columbus.

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THE VIRULENCE FOR MICE OF CERTAIN PNEUMOCOCCUS STRAINS FOLLOWING INDUCED SPECIFIC TYPE TRANSFORMATION. *Albert H. Harris,* Division of Laboratories and Research, New York State Department of Health, Albany.

The relationship of virulence to type specificity was studied by inducing reciprocal type transformation between a type of pneumococcus of high virulence for mice (type II) and one of low virulence for mice (type XIV). The technic was that described by Dawson and Warbasse. A strain representative of the transformation from type II to type XIV was tested for virulence, which was found to be as low as that of the type-XIV strain used in the preparation of the vaccine.

With some difficulty, a type-XIV strain was found which could be transformed to a type II. Virulence of this

[1] Aided by a grant from Eli Lilly and Company.

transformed strain was high, similar to the type-II strain from which the heat-killed vaccine was prepared.

These results strongly suggest that virulence is intimately associated with type specificity *per se*, at least as far as the strains of type II and type XIV which were employed are concerned, since, when the strains underwent specific type transformation, they assumed the virulence of the representative strain which was used in inducing the change to the new type.

THE ACTION OF SOIL MICROÖRGANISMS ON THE CARBOHYDRATES OF PNEUMOCOCCI OF TYPES I-XXXII. *Grace M. Sickles and Myrtle Shaw*, Division of Laboratories and Research, New York State Department of Health, Albany.

As an approach to the study of relationships between the various polysaccharides, comparative tests were made of the activity of *Bacillus palustris*, *Saccharobacterium ovale*, and *Saccharobacterium acuminatum* on the carbohydrates of pneumococci of types I to XXXII.

The polysaccharides of pneumococcal types, apparently related immunologically, were not always decomposed by the same strains of soil microörganism. The strain of *B. palustris* which produces an enzyme active against type-III carbohydrate decomposed type-III carbohydrate only. The very closely related strain of *B. palustris* which produces an enzyme that splits type-VIII carbohydrate decomposed that carbohydrate only. *S. ovale*, which was isolated on type-II carbohydrate medium, decomposed the polysaccharides of ten other types but not of type V. Two strains of *S. acuminatum* utilized the carbohydrates of twenty pneumococcal types. The polysaccharides of six types were decomposed by none of our strains.

The following cross reactions in precipitation tests disappeared simultaneously with the loss of precipitative reactivity in the homologous serum: type-II carbohydrate in types V and XX sera; type III in type-VIII serum; type VIII in types III and XIX sera; type XVI in type-XXVIII serum; type XXVIII in type-XVI serum; type XX in types X and XXXI sera, and type XXXI in type-XX serum.

PRELIMINARY REPORT ON THE ACTION OF IMMUNE SERUM IN CONJUNCTION WITH SULFANILAMIDE IN TYPE-III PNEUMOCOCCUS INFECTIONS IN MICE. *John K. Miller*, Division of Laboratories and Research, New York State Department of Health, Albany.

The effect of specific antibacterial serum, of sulfanilamide, and of serum combined with sulfanilamide therapy on pneumococcemia in mice was studied with 10 to 10,000 M.L.D. of a recently isolated and a stock mouse-passage strain of type-III pneumococcus. Sulfanilamide totaling 61.6 mg. was given to each animal, subcutaneously, over a period of five days: 17.6 mg. a day for two days and 8.8 mg. a day for three more days. Specific antibacterial rabbit serum alone in adequate doses afforded complete protection. With sulfanilamide alone, all animals were dead within ninety-six hours. Combined therapy with the above schedule gave variable results and in some experiments was not so effective as the use of serum alone.

However, when the schedule of dosage was varied so that 60 mg. of sulfanilamide were administered in two days, combined with serum which alone permitted 10-per cent survival, all animals survived approximately ninety-six hours, and 30- to 60-per-cent protection against the stock strain and 30 per cent against the more recently isolated strain was obtained. With

sulfanilamide alone, there was 100-per-cent mortality in ninety-six hours, but life was prolonged forty-eight hours longer than in the controls.

A Study of Meningococcal Cultures from Horses Immunized Against Meningococci. *Sophia M. Cohen*, Division of Laboratories and Research, New York State Department of Health, Albany.

Meningococci were isolated from eleven horses which developed endocarditis during immunization against several group I-III and group-II meningococcal strains which varied widely in virulence and other biologic characters. Forty-seven cultures were recovered from the blood or from lesions on the heart valves.

All cultures from ten horses were identified as group I-III; that from the eleventh, isolated only at autopsy, was mixed but group-II microörganisms predominated. No changes were observed in the agglutinative or precipitative activities of the meningococci isolated repeatedly from individual horses during the course of the infection. Those from eight of the horses showed a striking similarity to one particular stock group I-III strain used in immunization, which suggested that this strain is exceptionally well-adapted to survive and grow in the tissues.

The cultures studied from four of the horses exhibited a low degree of virulence for mice; those from the fifth a high degree, but after treatment of this animal with sulfanilamide a decrease in virulence was noted. Capsular swelling of the cultures tested was slight or absent as in the case of certain of the stock strains; viability in sodium-chloride solutions, in general, was marked. Cultures with these properties may possibly represent a phase in the dissociation of the meningococcus.

Purification and Analysis of Diphtheric Toxin by Ultrafiltration. *Mary W. Wheeler*, Division of Laboratories and Research, New York State Department of Health, Albany.

With the strain of diphtheria bacillus used in these studies—No. 3203—toxins with a flocculation value of from 10 to 14 and an M.L.D. of from 0.003 to 0.004 cc. were regularly produced in a synthetic medium in which the only sources of nitrogen added were aspartic acid and cysteine hydrochloride.

By ultrafiltration of culture filtrates through 8.5- or 9.5-per-cent Parlodion membranes, it was possible to obtain purified, concentrated residues which contained practically all of the proteins and toxin synthesized by the original culture. The nitrogen content varied from 0.001 to 0.002 mg. per Lf.

These residues could be separated by refiltration into two fractions: one, the portion retained by a 4.5-per-cent Parlodion membrane; the second, the residue obtained by reconcentrating on a 9.5-per-cent membrane the filtrate and washings from the 4.5-per-cent membrane.

The first fraction was relatively nontoxic and contained the proteins precipitable with 33-per-cent saturation of ammonium sulfate. The second fraction contained from 50 to 70 per cent of the toxin. The proteins present were precipitated with from 50- to 60-per-cent saturation with ammonium sulfate, but no precipitate was obtained with 33-per-cent saturation. The nitrogen content approximated 0.0005 mg. per Lf.

Further Observations on the Toxigenic Properties of Hemolytic Streptococci. *Julia M. Coffey*, Division of Laboratories and Re-

search, New York State Department of Health, Albany.

Five hundred and ninety-seven cultures of hemolytic streptococci were classified according to precipitation groups and examined for toxigenic activity; with 450 the virulence for mice was determined. The highest incidence of virulent strains occurred in group C-G. Although toxigenic activity as demonstrated by the cutaneous reactions induced in rabbits was largely limited to group A, a small number of group C-G cultures from horses produced toxins.

The toxigenic strains were divided into groups on the basis of toxin neutralization by univalent horse sera of three selected group-A strains. These sera alone or in combination neutralized the toxins of all of the strains of groups A and C-G. A multivalent horse serum produced with two strains of different toxin groups neutralized the toxins of 147 group-A strains which included representatives of the various toxin groups.

This study reemphasizes the complex character of the antigenic activity of streptococcus toxins. Within one toxin group marked differences were demonstrated by neutralization tests with several heterologous antitoxins and by studies of antigenic activity in goats and rabbits. The number of toxin groups defined is thus dependent upon the valency of the antitoxins used in classification. Furthermore, the antigenic valency of a toxin is in some instances modified by the animal species in which the antitoxin is produced.